

Vol. 49 No. 6

June 1961

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR
THE JAPANESE BIOCHEMICAL SOCIETY

EDITORIAL BOARD

SHIRO AKABORI	KATASHI MAKINO	NOBUO TAMIYA
SHICHIRO AKIYA	BUNJI MARUO	KYOSUKE TSUDA
TOSHIO ANDO	YOSHIAKI MIURA	TYUNOSIN UKITA
FUJIO EGAMI	TOMOO MIWA	ITARU WATANABE
SABURO FUNAHASHI	DEN'ICHI MIZUNO	YUICHI YAMAMURA
OSAMU HAYAISHI	ZIRO NIKUNI	TOMOMICHI YANAGITA
KOOZOO KAZIRO	NORIO SHIMAZONO	HARUHISA YOSHIKAWA

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

The University of Tokyo, Tokyo

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per 6 months.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides, original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥ 2,700 (U.S. \$7.50) per volume, postage inclusive. Back issues are available for sale, from volume 1 to volume 46 at ¥ 3,600 (U.S. \$10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co., Ltd.*, Chuo-ku, Tokyo, Japan.

AGENCIES

Charles E. Tuttle
Rutland, Vt.
U. S. A.

Maruzen Co., Ltd.
Tokyo
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

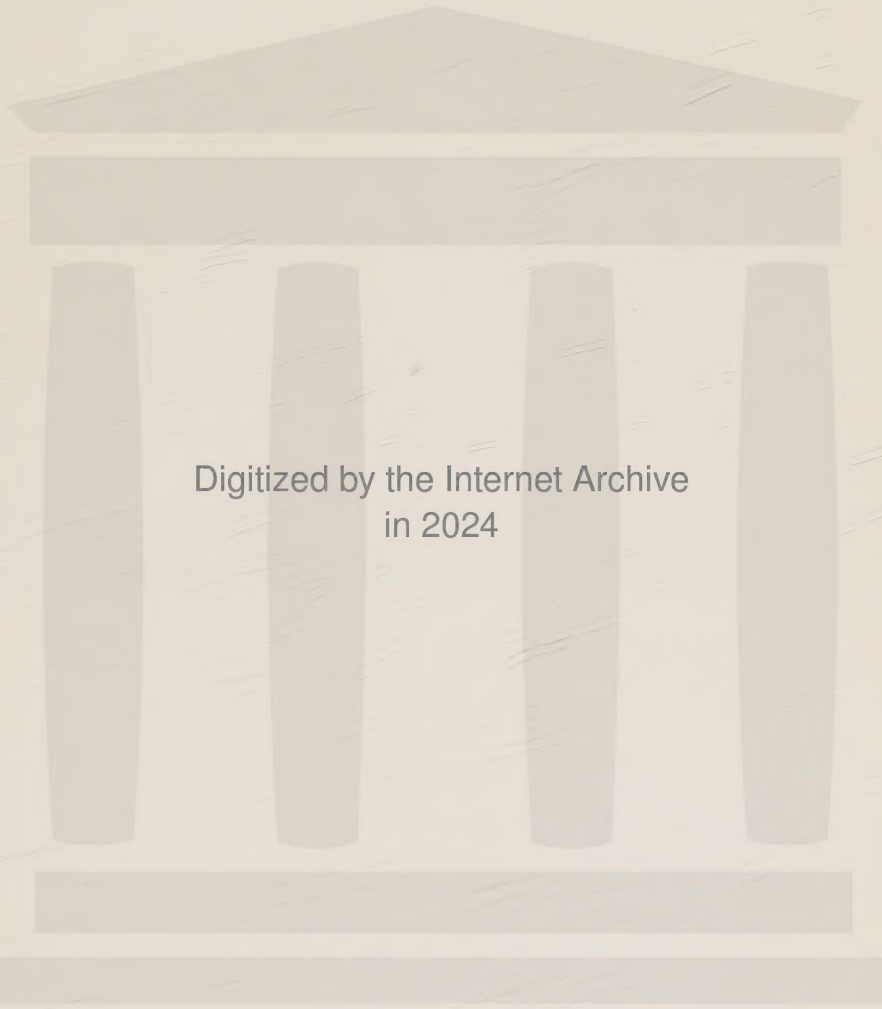
THIS NUMBER
OF THE JOURNAL OF BIOCHEMISTRY

CONTAINS A COLLECTION OF PAPERS DEDICATED TO

KEIZO KODAMA

ON THE OCCASION OF HIS 70TH BIRTHDAY

THE JAPANESE BIOCHEMICAL SOCIETY



Digitized by the Internet Archive
in 2024



K. Kodama

KEIZO KODAMA

On the Occasion of His Seventieth Birthday

June 17, 1961

Professor KEIZO KODAMA was born in Hikone, a small town near Kyoto, Japan, on June 17, 1891. He graduated from the Faculty of Medicine, Tokyo Imperial University (the University of Tokyo) in 1918. After nine years of graduate study in the Department of Biochemistry, under the direction of Professor S. KAKIUCHI at the University, he was appointed Professor of Biochemistry at Aichi Medical College as the successor to Professor L. MICHAELIS.

From 1924 to 1926, he stayed in England to study physical chemistry in the laboratory of Professor F. DONNAN in London and enzymology in the laboratory of Professor F. G. HOPKINS in Cambridge. In 1928, he was appointed Professor of Biochemistry at Kyushu Imperial University, School of Medicine.

In 1944 he returned to Tokyo Imperial University (the University of Tokyo) as Professor of Biochemistry. In the following years, he added to his talents as an administrator in the post of Dean of the Faculty of Medicine, the University of Tokyo, from 1949 to 1951.

At sixty, in 1952, he retired from the University of Tokyo and was appointed President of Tokushima University where he has spent the greater part of his time in the organization of this new University. In 1957, when an International Symposium on Enzyme Chemistry was held in Tokyo and Kyoto under the auspices of the International Union of Biochemistry, he contributed greatly to this meeting as the Chairman of the Organizing Committee.

His contributions to the development of biochemistry in Japan were truly great. It would be superfluous here to give an extended list of his papers or the many honors received by him. It must be emphasized, however, that one of his most noteworthy endeavours was the restoration and extension of the Japanese Biochemical Society while he held the presidency of the Society from 1945 to 1953.

One of his achievements was the re-activation of the Journal of Biochemistry which had been discontinued since 1944. Up to that year the Journal had been published by Professor SAMURO KAKIUCHI but the outbreak of World War II deprived the Journal of its international significance. Professor KODAMA, after the reorganization of the Japanese Biochemical Society in 1948, decided to republish the Journal under the sponsorship of the Society to introduce, throughout the world, the biochemical works done in Japan. In spite of great difficulties in the post-war period, the new issue appeared in June 1950 following the serial number of the discontinued to grow and to be read more and more widely throughout the world.

On the occasion of his seventieth birthday, this number of the Journal of Biochemistry is dedicated to Professor KODAMA with a deep sense of gratitude from all the members of the Japanese Biochemical Society.

NORIO SHIMAZONO

HARUHISA YOSHIKAWA

The Estimation of Serum Proteins by Ultraviolet Densitometry of Agar Electrophoretic Diagram

By KAZUO SHIMAO

(From the Faculty of Pre-medical and Pre-dental Courses,
Tokyo Medical and Dental University)

(Received for publication, September 10, 1960)

The use of agar as a supporting medium for electrophoretic analysis of proteins has been reported by several authors (1-5). Agar electrophoretic diagram being transparent to visible light, the method is said to be suitable for densitometric analysis after staining protein fractions by dye, such as amido black 10B (AB10B). As dried agar film is transparent not only to visible light but also to ultra-violet (UV) ray, the author tested the possibility of direct densitometric quantitation of agar electrophoretic diagram without staining procedure, and the results are reported in the present paper.

EXPERIMENTAL

Apparatus—Apparatus for agar electrophoresis is shown in Fig. 1. It is similar to those used by other authors (1, 2). Optical density of dried agar was determined by Model EPU-2 Hitachi photoelectric

spectrophotometer with the Type K-2 paper densitometry attachment.

Buffer Solution—Barbital sodium-hydrochloric acid buffer, pH 8.6 and ionic strength 0.05, was used.

Buffered Agar Gel—One per cent agar (Kyokuto Seika Ltd. Purified agar-agar) gel in the above mentioned buffer was used as a supporting medium for agar electrophoresis.

Fixing Solution—Nine volumes of ethanol was mixed with one volume of glacial acetic acid and used for fixation of protein fractions in agar gel.

Rinsing solution—Five per cent acetic acid solution was used.

Procedure for Agar Electrophoresis and UV Densitometric Analysis—10×28 cm. clean cellophane paper was immersed in the buffer solution, it was then adhered onto the bottom glass plate compactly by the ball of the finger leaving no creases nor bubbles between cellophane and glass plate. After setting a plastic frame of internal size 7×22 cm. on the cellophane, 20 ml. of 1% hot agar were poured onto the bottom

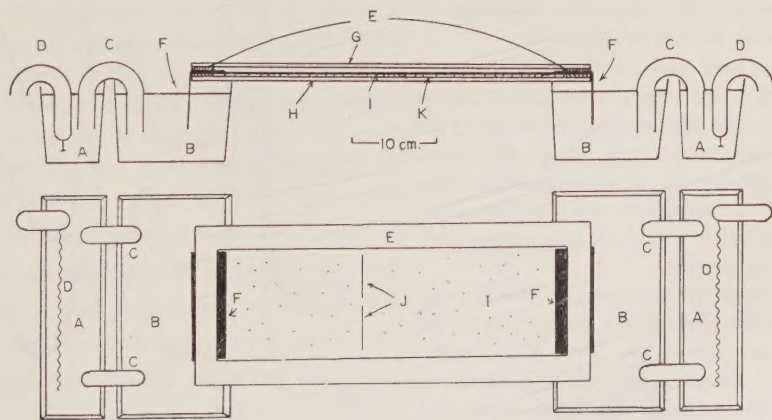


FIG. 1. Agar electrophoresis apparatus.

- | | |
|-------------------------|----------------------------|
| A: Electrode vessel | F: Connecting filter paper |
| B: Buffer vessel | G: Cover glass plate |
| C: Buffered agar bridge | H: Bottom glass plate |
| D: Platinum electrode | I: Buffered agar gel |
| E: Plastic frame | J: Cellophane |

glass plate to make agar gel of about 1 mm. thickness. When the gelification was complete, 0.025 to 0.030 ml. of serum was applied uniformly from capillary pipette on a filter paper strip of size 2×30 mm. The strip was then put on the gel 2 cm. to cathodic side from the middle of the gel. Gel and buffer vessels were connected by buffer saturated filter paper, and the second plastic frame and cover glass plate were set in position as shown in Fig. 1. 1.5 to 2.0 mA of current per cm. width of the gel was applied for about 4 to 5 hours. Serum albumin migrated 5 to 6 cm. anodically and γ -globulin 2 to 3 cm. cathodically under the above mentioned conditions at a room temperature of 15 to 20°C. When electrophoretic separation was completed, agar gel, with the bottom plate and cellophane, was dipped in about 200 ml. of the fixing solution, and the protein fractions were fixed for 60 minutes. Protein fractions could be seen as white precipitation bands in the gel at this stage of the procedure. The gel with the bottom plate was then rinsed with *ca.* 200 ml. of 5% acetic acid for 15 minutes with occasional shaking. The procedure was repeated three times. Finally, the gel was rinsed in *ca.* 500 ml. of the rinsing solution for 5 to 6 hours with occasional shaking. The gel leaved from cellophane during the rinsing process. A clean sheet of cellophane of the same size as before was immersed in 5% acetic acid solution and was put on a clean glass plate of the same size as the bottom glass plate leaving no creases nor bubbles between glass and cellophane. The gel was carefully taken on the acetic acid moistened cellophane, and a plastic frame was set on the cellophane on the glass plate by clips in order to prevent nonuniform shrinkage of the cellophane during the drying procedure.

The gel on the glass plate was put into electric oven at 40 to 50°C containing a water filled dish to prevent over-drying and the resulting breakage of the cellophane. Drying process took about six hours. After drying, the gel became a transparent uniform film on the sheet of cellophane on the glass plate. White precipitation bands of the proteins disappeared at the end of drying process.

The dried cellophane was cut into the size suitable for the apparatus (3×15 cm.), pulled away from the glass plate, and set in the Type K-2 densitometric attachment. Densitometry was carried out at a wave length of 280 $m\mu$ with the band width of 1 $m\mu$. In the present apparatus, a pitch of densitometry was 2 mm. and the effective size of the slit was 2×5 mm.

UV Absorption of Serum Proteins in Dried Agar Film—Buffered agar gel of known thickness (1 mm.)

was cut into two pieces. One of them was immersed in the protein solution of known concentration, the other in the buffer solution for 48 hours. After equilibration, both gel pieces were treated as mentioned above, and optical density of the dried agar gel containing protein was read against that containing no protein. Surface concentration of protein was calculated from the volume and thickness of the gel and the volume of the protein solution. $E_{280m\mu}^{0.01g/cm^2}$ of the protein was calculated from the results obtained.

UV absorption of serum proteins in solution—UV absorption of serum proteins in solution was determined in phosphate buffer, pH 7.7 and ionic strength 0.1, with Model EPU-2 Hitachi spectrophotometer.

Free Electrophoresis—Free electrophoresis was performed using barbital sodium-hydrochloric acid buffer, pH 8.6 and ionic strength 0.1.

Agar electrophoretic analysis of serum proteins using AB10B staining procedure was performed by the method reported in the previous publication (6).

RESULTS

Electrophoretic diagrams obtained by repeated analyses of the same serum are shown

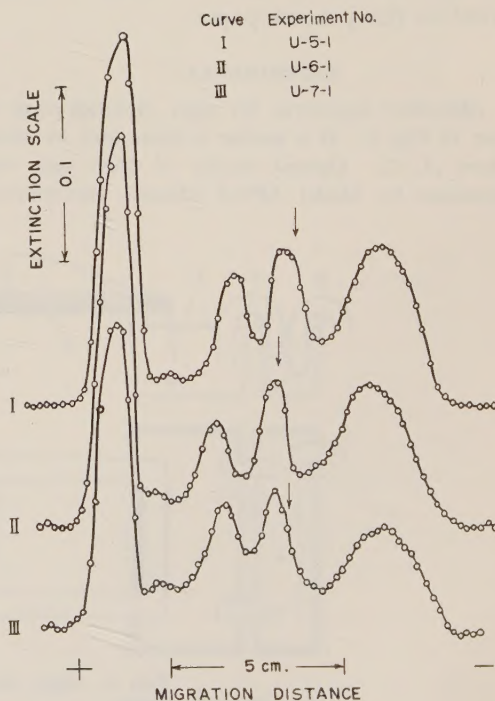


FIG. 2. Comparison of electrophoretic diagrams obtained by triplicate experiments on the same serum. Corresponding protein values are shown in Table I.

TABLE I
Reproducibility of Serum Protein Values Obtained by
the Present Procedure

Material	Experiment No.	Albumin %	Globulin			
			α_1 %	α_2 %	β %	γ %
Serum A	U-5-1	35.2	2.6	13.2	17.7	31.4
	U-6-1	35.4	2.3	11.4	14.9	36.0
	U-7-1	32.2	3.5	15.6	16.2	32.4
	U-10-A	35.3	3.1	13.6	17.6	30.6
	Mean $\pm \sigma$	34.5 \pm 1.6	2.9 \pm 0.6	13.5 \pm 1.7	16.6 \pm 1.4	32.6 \pm 2.1
Serum T	U-5-2	39.3	3.3	16.6	20.9	19.9
	U-6-2	42.0	2.6	16.6	18.3	19.7
	U-7-2	39.8	2.6	18.5	18.5	20.6
	Mean $\pm \sigma$	40.4 \pm 1.4	2.8 \pm 0.5	17.2 \pm 1.0	19.2 \pm 1.5	20.1 \pm 0.5
Albumin- γ -globulin mixture A	U-3-1	49.0				51.0
	U-4-1	48.8				51.2
	U-8-1	46.9				53.1
	Mean $\pm \sigma$	48.2 \pm 1.3				51.8 \pm 1.3
Albumin- γ -globulin mixture B	U-3-2	61.2				38.8
	U-4-2	57.2				42.8
	U-8-2	61.8				38.2
	Mean $\pm \sigma$	60.1 \pm 2.1				39.9 \pm 2.1

σ : standard deviation

in Fig. 2, corresponding values and the values from other experiments are listed in Table I. From the figure and the table, reproducibility of the procedure seems satisfactory. UV absorption at $280\text{ m}\mu$ of serum and serum protein fractions in solution or in agar film is shown in Table II and Fig. 3. UV absorption in dried agar film of human serum obeyed Beer's law. $E_{280\text{ m}\mu}^{1\%, 1\text{cm.}}$ in solution was almost the same as $E_{280\text{ m}\mu}^{0.01\text{g./cm}^2}$ measured on dried agar film for both serum albumin and γ -globulin as seen in Fig. 3. $E_{280\text{ m}\mu}^{1\%, 1\text{cm.}}$ for the both protein fractions were slightly larger than those reported in literature (7, 8). This was so, because $E_{280\text{ m}\mu}^{1\%, 1\text{cm.}}$ values in Table II were not corrected for turbidity of the solution.

$E_{280\text{ m}\mu}^{1\%, 1\text{cm.}}$ values for serum protein fractions obtained by starch zone electrophoresis or

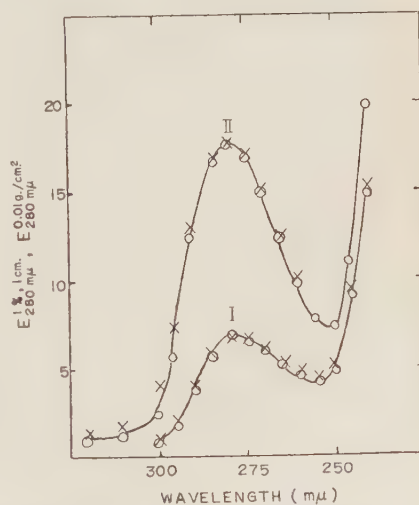


FIG. 3. UV absorption of human serum albumin (I) and γ -globulin (II) in solution (○) and in dried agar gel (×).

TABLE II

UV Absorption of Human Serum Proteins in Solution and in Dried Agar Gel

Material	UV absorption in solution		UV absorption in dried agar gel		Method of preparation ¹⁾
	Concentration mg./ml.	$E_{280m\mu}^{1\%, 1cm.}$ at pH 7.7	concentration mg./cm ² .	$E_{280m\mu}^{0.01 g/cm^2.}$	
Albumin	3.96	6.70	0.333	6.45	C
	2.22	6.71			S
	3.6	6.3			Z
γ -Globulin	2.55	17.4	0.246	17.3	C
	0.25	17			S
	1.47	15			Z
α -Globulin	0.57	13			Z
β -Globulin	0.82	20			Z
Pseudo-Globulin-I	0.24	14			S
Pseudo-Globulin-II	0.20	12			S
Serum A	5.30	10.0	0.075	10.4	
			0.147	10.1	
			0.224	10.1	
			0.298	10.2	
			0.372	10.2	
			0.447	10.1	
			0.522	9.8	

1) C: Commercial sample, S: Salting out with sodium sulfate,

Z: Starch zone electrophoresis, using phosphate buffer, pH 7.8 and ionic strength 0.075. Salting-out and zone electrophoresis were carried out using serum A.

TABLE III

Comparison of Data Obtained by Different Method of Electrophoresis

Material	Method ¹⁾	Composition of serum proteins				
		Albumin %	Globulin			
			α_1 %	α_2 %	β %	γ %
Serum A	UV	31	3	7	27	32
	T	52	4	7	13	24
	AB10B	78	1	2	8	11
Serum T	UV	26	5	22	11	36
	T	37	6	18	12	27
	AB10B	67	2	10	4	17
Serum AN	UV	10	2	4	5	79
	T	12	5		7	77
	AB10B	19	1	1	1	78

1) UV: Direct densitometry of the present paper T: Free electrophoresis with Tiselius apparatus AB10B: Densitometry after staining with AB10B.

sodium sulfate salting-out are also shown in Table II. $E_{280m\mu}^{0.01 g/cm^2.}$ for protein fractions other than albumin and γ -globulin was not determined, but it might be nearly the same as $E_{280m\mu}^{1\%, 1cm.}$ of each fraction, when we refer to the results shown in Table II and Fig. 3 for serum albumin and γ -globulin. In Table III, the results of electrophoretic analysis of serum by three different methods are shown. Albumin values were the highest for AB10B staining procedure and the lowest for UV densitometry.

DISCUSSION

UV absorption has been used by various authors for the detection of proteins or other substances on filter paper in paper electrophoresis or chromatography (9-11). UV absorption of proteins was determined by Louf-bourow (9) after drying down suspension on quartz plate. Svensson (12) made use

of UV absorption for the estimation of protein concentration in density gradient electrophoresis. It seems to the author that no attempt has been made to use direct UV densitometry for the estimation of serum proteins in electrophoresis using supporting medium. The results reported here shows that by combination of UV densitometry with agar electrophoresis, a new procedure for the determination of serum protein fractions is possible. Protein fractions fixed by ethanol-acetic acid mixture in agar gel and dried down on cellophane was transparent and UV absorption at $280\text{ m}\mu$ obeyed Beer's law as shown in Table II. Barbitol being rinsed out of agar gel during fixation and rinsing process, UV absorption of buffer did not hinder UV densitometry at all. Transparency of dried agar gel on cellophane against air was found to be between 85 and 90 per cent. Percentage values for serum protein fractions obtained by the present method were, of course, different from that obtained by free electrophoresis or other method as shown in Table III. Difference between values obtained by the present method and free electrophoresis could be explained by the difference between fractions of $E_{280\text{ m}\mu}^{0.01\text{ g./cm.}^2}$, which were nearly the same as $E_{280\text{ m}\mu}^{1\%, 1\text{ cm.}}$ of each fraction in solution. When values of UV densitometry for serum A in Table III are corrected for $E_{280\text{ m}\mu}^{1\%, 1\text{ cm.}}$ of protein fractions of Table II, percentage values of albumin + α_1 -globulin, α_2 -, β -, and γ -globulins became, 58, 6, 15, and 22 per cent respectively, which are in fairly good agreement with those obtained by free electrophoresis.

The present method does not include any staining procedure, which has very often been a source of errors of analysis or of deviations among data from different laboratories obtained by the same procedure in paper electrophoresis. $E_{280\text{ m}\mu}^{1\%, 1\text{ cm.}}$ of albumin was much less than that of other globulin fraction, and the

fact resulted in the larger percentage values of globulin fractions, and reduced the relative error of percentage values of these fractions.

SUMMARY

A new method of agar electrophoretic analysis of serum protein was reported. Proteins separated by agar electrophoresis were fixed by ethanol-acetic acid mixture and dried down on a sheet of cellophane. By direct densitometry at $280\text{ m}\mu$ of the dried agar on cellophane, percentage values of each protein fraction was obtained. $E_{280\text{ m}\mu}^{0.01\text{ g./cm.}^2}$ values for albumin and γ -globulin fractions were almost the same as $E_{280\text{ m}\mu}^{1\%, 1\text{ cm.}}$ of each fraction in neutral solution. Because of the difference in $E_{280\text{ m}\mu}^{1\%, 1\text{ cm.}}$ of serum protein fractions, percentage values obtained by the present method were different from those obtained by free electrophoresis, but this difference could reasonably be explained by $E_{280\text{ m}\mu}^{1\%, 1\text{ cm.}}$ of serum protein fractions. The method seems to be useful as a new method of electrophoretic analysis of serum proteins.

REFERENCES

- (1) Giri, K. V., *J. Indian Inst. Sci.*, **38**, 190 (1956)
- (2) Giri, K. V., *Ibid.*, **39**, 224 (1957)
- (3) Giri, K. V., *Ibid.*, **39**, 248 (1957)
- (4) Giri, K. V., *Naturwiss.*, **43**, 36 (1956)
- (5) Uriel, J., and Grabar, P., *Bull. soc. chim. biol.*, **38**, 1253 (1956)
- (6) Shimao, K., *Nippon-Rinsho (Japan)*, **17**, 568 (1959)
- (7) Goodwin, T. W., and Morton, R. A., *Biochem. J.*, **40**, 628 (1946)
- (8) Lerner, A. B., and Barnum, C. P., *Arch. Biochem. Biophys.*, **11**, 505 (1946)
- (9) Holiday, E. R., and Johnson, E. A., *Nature*, **163**, 216 (1949)
- (10) Semm, K., and Fried, R., *Naturwiss.* **39**, 326 (1952)
- (11) Kimbel, K. H., *Naturwiss.* **40**, 200 (1953)
- (12) Svensson, H., Hagdahl, L., and Lennen, K. D., *Scientific Tools*, **4**, 1 (1957)

Role of Mitochondria in the *in vitro* Formation of Protoporphyrin and Haem

By C. RIMINGTON and B. E. TOOTH

(From the Department of Chemical Pathology, University College Hospital Medical School, London, England)

(Received for publication, January 30, 1961)

Although the broad outline of the pathway of haem biosynthesis is now firmly established (1), certain points of detail remain to be elucidated. In particular, the transformation of coproporphyrinogen to protoporphyrinogen or protoporphyrin, involving oxidative decarboxylation of two carboxyethyl side chains (positions 2 and 4) to yield vinyl groups and the mechanism of insertion of iron into the porphyrin ring to form haem are still poorly understood. Both steps appear to be enzymically controlled but the number of enzymes involved and whether or not co-factors are necessary are unknown.

That mammalian reticulocytes are capable of synthesizing haem *in vitro* was first demonstrated by Reimann (2), in 1942 and has since been amply confirmed (3, 4). These cells contain mitochondria (5-8) as do also the synthetically-active basophilic stippled cells of lead poisoned animals (9). Sano (10) has shown that mitochondria participate at three stages in the biosynthesis of haem, namely (1) in the formation of δ -aminolaevulinic acid (ALA) from glycine and active succinate (11-14), (2) in some step or steps in the synthesis of protoporphyrin from ALA and (3) in the incorporation of iron into the porphyrin ring. He found that in stage (2) an extract prepared from an acetone powder was as effective as intact mitochondria from rabbit liver, chicken erythrocytes, bone marrow or mesenteric lymph glands. Mitochondria from rat kidney, heart, muscle and intestinal mucosa were inactive.

FORMATION OF PROTOPORPHYRIN

Falk, Dresel and Rimington (15)

showed that haemolysates of chicken red cells produced large quantities of protoporphyrin and smaller amounts of coproporphyrin and uroporphyrin from added porphobilinogen (PBG). The supernatant fraction derived from centrifugation of such haemolysates ($20,000 \times g$ for 20 minutes) formed only uro- and coproporphyrins but protoporphyrin production was restored by adding a rat liver mitochondrial preparation. Mitochondria alone produced no porphyrin (16). Haemolysates of human red cells were found by Rimington and Booiij (17) to form only traces of protoporphyrin from PBG but, here again, addition of liver mitochondria raised the yield fifteen or twenty fold. A system or fraction present in mitochondria appears to be necessary to complete the protoporphyrin-forming system of red cell haemolysates. Falk, Dresel and Rimington (15) found that oxygen was necessary for protoporphyrin production by the whole haemolysate of chicken red cells from PBG. Replacement of air by nitrogen markedly lowered the yield of this porphyrin suggesting that oxygen was involved as a hydrogen acceptor in the overall oxidative decarboxylation reaction.

The experiments which we now report have extended over the last two years and have been directed towards a more detailed elucidation of the role of mitochondria in systems derived from human and chicken red blood cells or from *Rhodospseudomonas spheroides*.

METHODS

Chicken Red Cell Haemolysate (CH)—This was prepared according to Dresel and Falk (18).

Chicken Red Cell Enzyme (CBZ)—Heparinized

chicken blood (10 ml.) was centrifuged at approximately $2000\times g$ for 10 minutes at 0°C , plasma removed and the cells suspended in 0.9% NaCl. After centrifuging as before, the saline and white cell layer was removed and the cells washed once more by 0.9% NaCl. Benzene (1 ml.) was added to the red cell mass which was shaken until haemolysis was complete (1 minute). An equal volume of glass-distilled water was then added and after thorough mixing by shaking for 1 minute, the enzyme-containing supernatant was separated by centrifuging for 10 minutes at room temperature and removing the benzene layer. It was filtered through paper and stored at 5°C . This clear preparation yielded no deposit when it was centrifuged for 15 minutes at $11,750\times g$.

Preparation of Mitochondria—The weighed liver of a freshly killed rat was homogenised in 0.25 molar sucrose (10% w/v). After centrifuging at $2000\times g$ for 10 minutes at 5°C , the supernate was removed and centrifuged at $11,750\times g$ for 15 minutes at 0°C . The residue was suspended in 0.25 molar sucrose and again centrifuged under the same conditions. The final residue was suspended in a volume of glass-distilled water equal to half the weight of liver used and stored at -10°C .

Mitochondrial Supernatant—The suspension of mitochondria was frozen and thawed three times then centrifuged at $11,750\times g$ for 15 minutes at 0°C and the supernatant collected.

Acetone Powder of Mitochondria—A suspension of mitochondria in glass-distilled water (2 ml.) was shaken with ice-cold acetone (40 ml.) and left to stand at -10°C for 30 minutes. The mixture was filtered in the cold on a Buchner funnel and the residue washed with 20 ml. of ice-cold acetone, then dried in a vacuum desiccator. To prepare an extract, the powder was suspended in glass distilled water (2 ml.), ground up thoroughly and the supernatant recovered after centrifuging at $11,750\times g$ for 15 minutes.

Coproporphyrinogen—A strong solution (2 ml.) of coproporphyrin III in 0.2 *N* NaOH was shaken in a glass-stoppered flask wrapped in black cloth with 4 g. freshly ground sodium amalgam for 5 minutes. The colourless solution was then filtered rapidly with the aid of suction through four layers of Whatman No. 42 filter paper on a sintered glass funnel into a small test tube. The pH was cautiously adjusted to approximately 7.5.

Porphobilinogen (PBG)—This was dissolved in phosphate buffer pH 7.4 (1 ml. of 0.1 molar KH_2PO_4 + 4 ml. of 0.1 molar Na_2HPO_4).

Incubation—Small test-tubes were immersed in a water-bath at 37°C for the times and under the con-

ditions stated. For anaerobic experiments, evacuated Thunberg tubes were used containing alkaline pyrogallol in the side arm. Experiments were conducted in subdued light or darkness.

Analytical Methods—The contents of the tubes were mixed with ethyl acetate: acetic acid (3:1) and centrifuged for 5 minutes at $2000\times g$. After removal of the supernatant into a small separatory funnel, the residue was washed repeatedly on the centrifuge with the acid mixture until the supernatant was no longer fluorescent. The bulked supernatants were shaken twice with saturated sodium acetate and these washings re-extracted by ethyl acetate until the latter was no longer fluorescent. It was then added to the main extract which was shaken once with 3% sodium acetate. Total porphyrin was then extracted by shaking with 15% HCl leaving haem in the organic phase. In experiments using Fe^{59} , this was made up to 25 ml. and the radioactivity counted in a well-type scintillation counter.

The 15% HCl solution containing the porphyrins was neutralized to congo red with saturated sodium acetate and the porphyrins extracted by repeated shaking with ether. After one wash by 3% sodium acetate followed by one with water, the ether was shaken with small quantities of 0.36% HCl until all coproporphyrin was removed and then with 5% HCl to remove protoporphyrin. Optical density of each extract was then determined at $380\text{ m}\mu$, $430\text{ m}\mu$ and at the Soret maximum (D_{max}) using 1 cm. cells and a Unicam photoelectric spectrophotometer. Porphyrin content was calculated using the correction formula of Rimington and Sveinsson (19), viz:

$$\mu\text{g. Copro.} = (2D_{\text{max}} - D_{380} - D_{430}) \times \frac{1.5}{1.833} \times V$$

$$\mu\text{g. Proto.} = (2D_{\text{max}} - D_{380} - D_{430}) \times \frac{2.04}{1.67} \times V$$

where *V* is the volume of the extract in each case.

Control experiments showed that any porphyrinogen originally present was oxidized to porphyrin during the manipulations and included in the final extract.

Fe^{59} —This was obtained from the Radiochemical Centre, Amersham, and used as FeCl_3 diluted with sterile 0.9% NaCl.

All reagents were of analytical grade and only glass-distilled water was used.

RESULTS

Analytical Recovery experiments—Some absorption with consequent loss of porphyrin upon precipitated protein occurred whatever

precipitant was used. Acetone was perhaps somewhat superior to the ethyl acetate: acetic acid mixture but was less convenient in fur-

TABLE I

Recovery of Coproporphyrin Added to Enzyme Preparation CBZ

Porphyrin added ($\mu\text{g.}$)	Recovery (%)	Conditions
13.60	94.9	No enzyme added.
13.60	94.2	No enzyme added.
5.44	88.3	No enzyme added.
5.44	86.4	No enzyme added.
5.44	81.5	Precipitated immediately.
5.44	76.8	Precipitated immediately.
5.44	71.0	Precipitated immediately and left overnight at room temperature.
5.44	71.7	
3.28	66.0	Precipitated immediately.
3.28	72.2	Precipitated immediately.
3.28	66.7	Acetone used as precipitant.
3.28	74.6	Acetone used as precipitant.
4.00	71.8	Acetone: HCl used as precipitant.

ther working up. Thorough washing of the protein precipitate with the acetate mixture and re-extraction of the different phases, as described under Methods, minimized such losses, and no correction was applied since all experiments were comparative. Percentage recoveries were materially greater when larger quantities of coproporphyrin were employed. Some typical results are included in Table I.

Porphyrins Formed from Porphobilinogen (PBG) by Different Enzyme Preparations—Chicken red-cell haemolysate contains all the enzymes necessary for the synthesis of haem from glycine. When δ -aminolaevulinic acid or porphobilinogen is used as substrate, in addition to haem much protoporphyrin is produced together with lesser quantities of coproporphyrin. A similarly prepared haemolysate from human erythrocytes and PBG forms very little protoporphyrin unless mitochondria are also added. The preparation made with benzene from chicken red-cells, CBZ, also retains the enzymes necessary for the utilization of PBG but porphyrin production stops at the coproporphyrin stage unless mitochondria are added. Mitochondria alone produced

TABLE II

Properties of Different Enzyme Preparations Utilizing PBG as Substrate

Incubation for 15 hours at 37°C except when stated.

ENZYME PREPARATION				Mitochondria	PBG ($\mu\text{g.}$)	Copro. ($\mu\text{g.}$)	Proto. ($\mu\text{g.}$)
Chicken red-cell haemolysate, CH, 4 hrs.					100	1.44	8.32
Human	"	"			15	0.16	0.05
"	"	"		+	"	0.06	0.12
Rat	"	"			15	0.11	0.05
"	"	"		+	"	0.02	0.19
Chick red-cell, benzene method, CBZ					30	5.09	0.17
"	"	"	" (heated)		"	0.10	0.03
"	"	"	"	+	"	2.20	0.81
"	"	"	" (heated)	+	"	0.04	0.02
"	"	"	" (heated)	+(heated)	"	0.05	0.04
"	"	"	"	+(heated)	"	5.11	0.28
Human red-cell, benzene method					"	1.50	0.13
"	"	"		+	"	1.76	1.33
"	"	"	(heated)	+	"	0.04	0.05
"	"	"	(heated)		"	0.15	0.03

no porphyrin from PBG and heating destroyed their ability to complete the protoporphyrin-forming system. Some properties of the different enzyme preparations are recorded in Table II.

Properties of the Chick Red-Cell Preparation CBZ—The soluble enzyme system prepared from chick red cells by the benzene method was selected for further study. The optimum pH was found to be ~7.4. The time course

of coproporphyrin production from PBG (60 µg.) is illustrated in Fig. 1, whilst Fig. 2 shows the increase of coproporphyrin and protoporphyrin with time when mitochondria are added to the preparation.

The preparation is moderately stable, 80% activity being retained during 15 days at 5°C.

When the enzyme preparation was ultra-filtered and the activities of resuspended residue and of ultrafiltrate, respectively, were separately measured, all the activity was found to reside in the residue with no indication of the requirement of any filterable co-factor (Table III).

TABLE III

Formation of Porphyrins from PBG (30 µg.) by Non-filterable Residue and Ultrafiltrate of Enzyme Preparation CBZ

	Copro. (µg.)	Proto. (µg.)
Whole enzyme preparation	2.98	0.22
Non-filterable residue	3.32	0.54
Ultrafiltrate	0.02	0.02
Recombined fractions	2.57	0.17

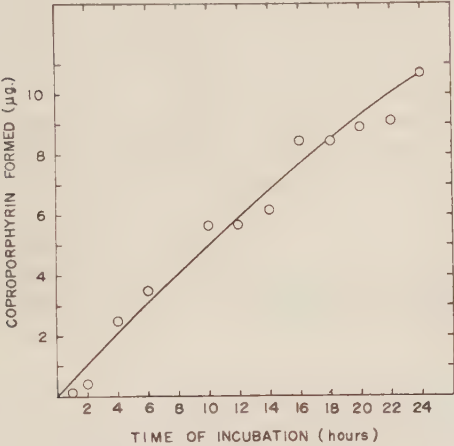


FIG. 1. Increase of coproporphyrin with time of incubation in the system: Chick red-cell enzyme CBZ+porphobilinogen (60 µg.).

Role of Mitochondria in Protoporphyrin forma-

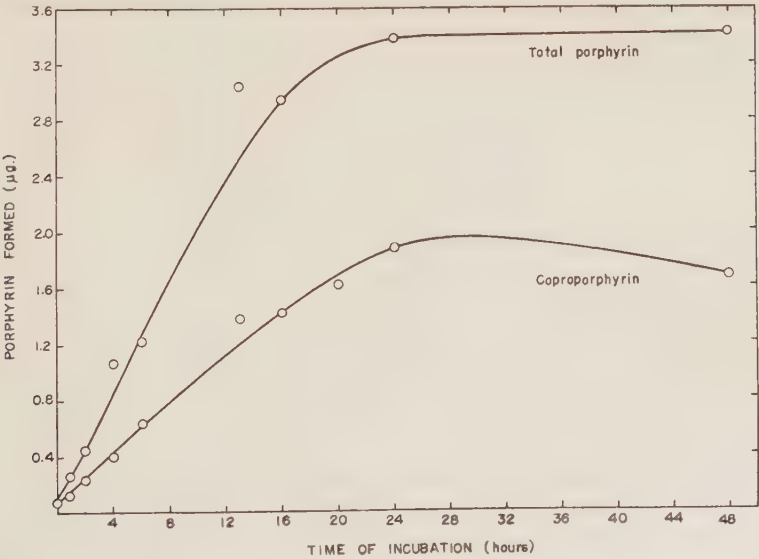


FIG. 2. Increase of coproporphyrin and total porphyrin with time of incubation in the system: Chick red-cell enzyme CBZ+rat liver mitochondria+porphobilinogen.

tion—The chick red-cell preparation CBZ made with the use of benzene carries porphyrin formation no further than to coproporphyrin. If rat liver mitochondria are added, the oxidative decarboxylation to protoporphyrin proceeds and it was thought that a closer study of this phenomenon might throw light upon the mechanism of the reaction.

Not all mitochondria were effective, those prepared from rat heart or kidney being inactive and rat spleen mitochondria only feebly active.

Liver mitochondria retain their activity during storage at -5°C for 8 weeks but are inactivated by 2 minutes heating at 100°C . Much of the activity is retained by an acetone powder preparation. Disruption by repeated freezing and thawing caused no loss of activity and after centrifuging at $11,750\times g$ for 15 minutes, activity was recovered in the supernatant. Ultrafiltration of the latter

showed that activity resided in the non-filterable residue; it was destroyed by heating to 100°C . These properties, which are illustrated in Table IV, indicate that the factor supplied by liver mitochondria is enzymic in nature and is not a thermostable co-factor.

It is now well established that the porphyrinogens rather than the porphyrins are the true intermediates in protoporphyrin and haem synthesis. Experiments were therefore carried out using coproporphyrinogen III as substrate with and without the addition of mitochondria or the supernate from frozen and thawed mitochondria.

From Table V it will be seen that the supernate was again as active as intact mitochondria in completing the protoporphyrin forming system.

In many enzymic systems, ferricyanide is able to replace oxygen as the final hydrogen acceptor; preliminary experiments were there-

TABLE IV
*Activity of Mitochondrial Fractions in Completing the System CBZ plus PBG
(app. 30 $\mu\text{g.}$) for Protoporphyrin Formation*

Preparation of fraction added	Copro. ($\mu\text{g.}$)	Proto. ($\mu\text{g.}$)
None	9.03	0.21
Whole mitochondria (rat liver)	6.74	2.69
Whole mitochondria (rat liver)	3.03	0.98
Acetone powder of above	3.63	0.76
Supernatant after freezing and thawing, 0.1 ml.	1.28	2.87
0.2 ml.	0.79	4.52
0.3 ml.	0.43	4.10
None	6.05	0.16
Supernatant of frozen and thawed mitochondria (rat liver)	2.49	1.65
Non-ultrafilterable residue of above	1.88	2.39
Ultrafiltrate	5.87	0.10
None	9.95	0.36
None	10.37	0.37
Whole mitochondria (rat heart)	10.00	0.29
Whole mitochondria (rat spleen)	2.52	0.33
"	2.85	0.55
" (rat kidney)	1.53	0.15
"	1.81	0.15

TABLE V

Formation of Protoporphyrin from Coproporphyrinogen III (C'GN) in Presence of CBZ and Rat Liver Mitochondria or a Supernate Prepared from Them by Freezing and Thawing

Enzyme system	Substrate	Copro. ($\mu\text{g.}$)	Proto. ($\mu\text{g.}$)
CBZ+rat liver mitochondria	PBG (30 $\mu\text{g.}$)	5.48	2.74
"	C'GN	5.74	1.01
Mitochondria only	"	7.06	0.71
None	C'GN	3.46	0.04
CBZ+rat liver mitochondria	"	2.42	1.16
Mitochondria only	"	3.14	0.09
CBZ+mitochondrial supernate	C'GN	25.20	4.26
Supernate only	"	26.00	0.55

TABLE VI

Effect of Some Inhibitors on Conversion of PBG (30 $\mu\text{g.}$) to Porphyrins by Preparation CBZ

Addition	Copro. ($\mu\text{g.}$)	Proto. ($\mu\text{g.}$)	Total ($\mu\text{g.}$)
None	3.33	0.43	3.76
K ferricyanide ($10^{-3} M$)	0.19	0.09	0.28
Liver mitochondria	4.3	5.9	10.2
" "	4.2	4.6	8.8
" " +NaF ($10^{-2} M$)	4.3	6.2	10.5
" " + "	4.2	6.2	10.4
" " + " ($5 \times 10^{-2} M$)	3.3	6.5	9.8
" " + "	3.4	6.7	10.1
None	2.44	0.18	2.62
Liver mitochondria	0.59	0.75	1.34
" " + iodoacetate ($10^{-3} M$)	0.16	0.92	1.08
" " + " ($5 \times 10^{-3} M$)	0.19	1.10	1.29
" " + " ($10^{-2} M$)	0.23	1.21	1.44
None	2.91	0.41	3.32
Liver mitochondria	0.53	1.30	1.83
" " + o-phenanthroline ($5 \times 10^{-3} M$)	0.39	2.39	2.78
" " + " ($10^{-2} M$)	0.55	1.70	2.25
" " + " ($2 \times 10^{-2} M$)	0.65	0.88	1.53
None Aerobic	4.58	1.74 ¹⁾	6.32
KCN ($5 \times 10^{-2} M$) Aerobic	2.91	2.79 ¹⁾	5.70
None Anaerobic	5.80	2.12 ¹⁾	7.92
KCN ($5 \times 10^{-2} M$) Anaerobic	4.68	3.08 ¹⁾	7.76

1) Uroporphyrin ($\mu\text{g.}$)

fore carried out in which this substance was added to the CBZ system. Potassium ferricyanide at $10^{-3} M$ concentration had a generally inhibitory effect, probably due to oxidation by it of coproporphyrinogen to coproporphyrin as the latter was formed in the system from the substrate (PBG). Ferricyanide could not, therefore, replace rat liver mitochondria.

The action of some other substances is also recorded in Table VI. Sodium fluoride was without significant effect up to a concentration of $10^{-2} M$. Since the CBZ preparation is capable of forming haem from protoporphyrin in the presence of mitochondria, one observes a decreased yield of total porphyrin when comparing experiments with and without addition of mitochondria although in the former case protoporphyrin production is considerably raised at the expense of coproporphyrin. Iodoacetate between $10^{-3} M$ and $10^{-2} M$ definitely enhanced protoporphyrin production.

The effect of cyanide was examined, aerobically and anaerobically on the CBZ preparation acting on PBG without added mitochondria and in these experiments yields of uroporphyrin and coproporphyrin only were determined, because Lockwood and Benson (20) have shown that in the presence of KCN this system forms coproporphyrinogen I; no further conversion to protoporphyrin would be expected since the enzymes responsible for this step act only on the III series isomer.

It will be seen from Table VI that KCN in a concentration of $5 \times 10^{-2} M$ had little effect upon the quantity of porphyrin produced from PBG except that the uro-copro conversion was slightly retarded both in aerobic and anaerobic conditions.

The most interesting effect upon the CBZ system was obtained with *o*-phenanthroline. Two actions appear to be discernable, an increase in the proportion of protoporphyrin to coproporphyrin formed but also a generally inhibitory effect, the total porphyrin yield decreasing progressively as the concentration of *o*-phenanthroline is raised.

FORMATION OF HAEM

The utilization of iron by red-cell haemolyzates to produce haem appears to be an enzymically catalysed reaction (21-24). Glutathione, ascorbic acid or cysteine act as co-factors (21, 23, 25) and it is probable that protoporphyrin rather than protoporphyrinogen is the immediate precursor (24).

The utilization of iron by intact avian erythrocytes and mammalian reticulocytes appears to follow a similar course (4) as does also haem synthesis by liver cell preparations (26-28).

There is, in addition, evidence suggesting that iron taken up by the reticulocyte is first bound to a protein constituent of the cell membrane from which it is released for haem formation (4, 29); binding to the microsomes may also be involved (30). Protoporphyrin may also react in a protein-bound form in haem synthesis (26, 28, 31).

A suitable system for investigation of the enzymic union of iron and protoporphyrin is provided by the chicken red-cell haemolyzate preparation CBZ, already described. Protoporphyrin was added in phosphate buffer, pH 7.4 (1 ml. of $0.1 M$ KH_2PO_4 and 4 ml. of $0.1 M$ Na_2HPO_4) and Fe^{59} as the citrate. After incubation at $37^\circ C$ under the conditions specified in each experiment, haem was extracted completely by successive treatments with ethylacetate-acetic acid [mixture (3:1)]. The combined extracts were treated as described under Methods and activity measured after dilution to 25 ml.; the necessary background and blank reading were recorded.

RESULTS

The chicken red-cell preparation CBZ had relatively feeble activity, but this was slightly increased by the addition of ascorbic acid ($10^{-3} M$) and markedly augmented by the addition of a suspension of liver mitochondria (Table VII).

As in the experiments upon protoporphyrin formation, the activity for haem formation was next tested of a supernate prepared by freezing and thawing rat liver

TABLE VII

Synthesis of Haem from Added Protoporphyrin and Fe⁵⁹ by Chicken Red-Cell Enzyme Preparation CBZ alone and in Presence of Ascorbic Acid or Rat Liver Mitochondria

CBZ	Mitochondria	Ascorbic acid (10 ⁻³ M)	Protoporphyrin	Fe ⁵⁹ added (c.p.m.)	Haem formed (c.p.m.)
+	+	0	+	7832	544
0	+	0	+	"	124
+	0	0	+	"	37
+	+	0	+	"	115
+	0	0	5.7 µg.	685	4
+	0	+	"	"	4
+	+	0	"	"	78
+	+	+	"	"	92
0	+	0	"	"	84
0	+	+	"	"	123
+	0	+	13.8 µg.	252	8
+	+	+	"	"	85

TABLE VIII

Comparison of the Activity of Rat Liver Whole Mitochondria with That of the Supernate Prepared from Them by Freezing and Thawing on Haem Synthesis in Presence of CBZ

Mitochondria	Mito. supernant	Substrate	Fe ⁵⁹ added (c.p.m.)	Haem formed (c.p.m.)
+	0	Protop.	7832	544
0	+	"	"	32
+	0	PBG (30 µg.)	"	528
0	+	"	"	106
+	0	"	"	935
0	+	"	"	113
+	0	"	"	938
0	+	"	"	130

TABLE IX

Haem-forming Activity of Rat Liver Mitochondria Preparations; Effect of Anaerobic Conditions and of Heating

System	Gas phase	Protop. (µg.)	Fe ⁵⁹ added (c.p.m.)	Haem formed (c.p.m.)
Rat liver mito.	Air	40	478	27
"	N ₂	"	"	204
" (heated)	Air	"	"	5
Rat liver mito. (whole)	Air	"	7832	124
" (supernate)	Air	"	"	4

TABLE X

Absence of Inhibition by Sodium Fluoride of Haem Synthesis by Rat Liver Mitochondria Using Either Protoporphyrin or Coproporphyrinogen as Substrate

Concentration of NaF (M)	Substrate	Fe ⁵⁹ added (c.p.m.)	Haem formed (c.p.m.)
0	Protop. (14.7 µg.)	10750	1150
10 ⁻²	"	"	1146
5×10 ⁻²	"	"	1132
10 ⁻¹	"	"	1110
0	Coproporphyrinogen	1075	47
10 ⁻²	"	"	35
5×10 ⁻²	"	"	37
10 ⁻¹	"	"	44

mitochondria three times and then centrifuging at 11,750×g for 15 minutes at 0°C. This was added to the CBZ preparation using either protoporphyrin or porphobilinogen as substrate. Compared with whole mitochondria, the supernate was virtually devoid of activity (Table VIII).

destroyed by heating and was favoured by anaerobic conditions (Table IX).

The optimum pH for haem synthesis, with protoporphyrin as substrate, was found to be 7.1 (Fig. 3). The time course of the reaction is illustrated in Fig. 4 which shows

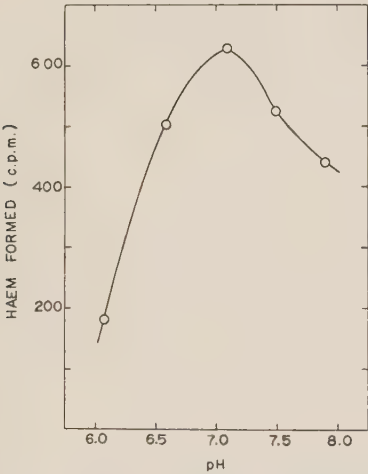


FIG. 3. Effect of pH on haem formation from protoporphyrin by rat liver mitochondria. System: Rat liver mitochondria+protoporphyrin (17.2 µg.)+ascorbic acid (10⁻³M)+Fe⁵⁹ (630 c.p.m.)+phosphate buffer.

All these experiments suggested that rat liver whole mitochondria alone were capable of catalysing efficient synthesis of haem from iron and protoporphyrin. A further series of experiments confirmed this; their activity was

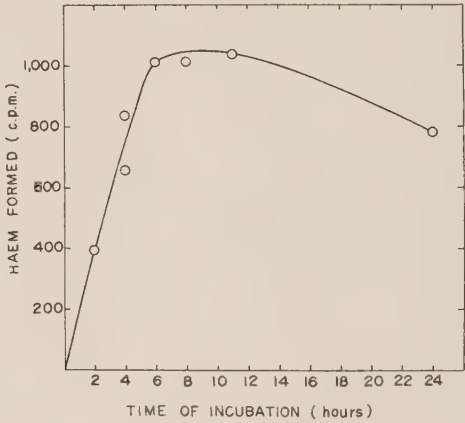


FIG. 4. Increase of haem formation with time of incubation in the system: Rat liver mitochondria+protoporphyrin (30 µg.)+Fe⁵⁹ (2983 c.p.m.).

a linear increase of haem during the first 6 hours. The effects of increasing iron and protoporphyrin respectively are shown in Figs. 5, 6 and 7.

Sodium fluoride was investigated for inhibitory activity but found to be without action at concentrations up to 10⁻¹M with either protoporphyrin or coproporphyrinogen as substrate (Table X).

The findings of Bénard, Gajdos and Gajdos-Török (32) with this substance can not, therefore, be attributed to an effect either upon the incorporation of iron into protoporphyrin or upon the oxidative decar-

boxylation of coproporphyrinogen to form protoporphyrin.

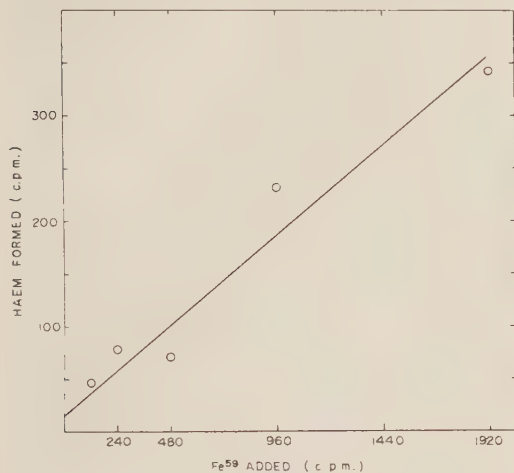


FIG. 5. Relation between haem formed and Fe^{59} added in the system: Rat liver mitochondria + protoporphyrin ($36.8 \mu\text{g.}$) + ascorbic acid ($10^{-3} M$).

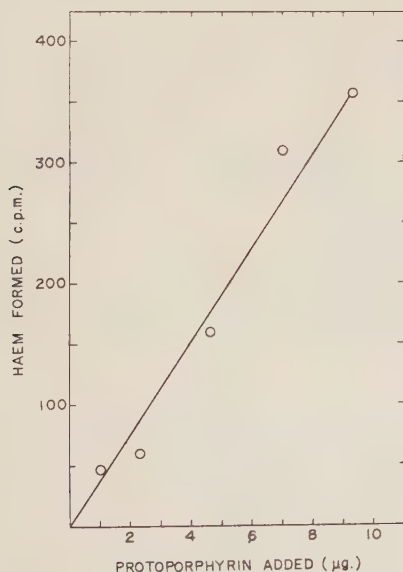


FIG. 6. Relation between haem formed and protoporphyrin added in the system: Rat liver mitochondria + Fe^{59} (768 c.p.m.) + ascorbic acid ($10^{-3} M$) + phosphate buffer pH 7.4 + protoporphyrin (up to $9.3 \mu\text{g.}$).

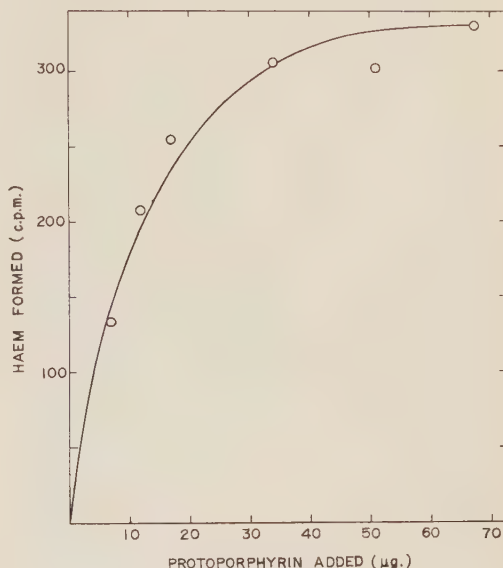


FIG. 7. Relation between haem formed and protoporphyrin added in the system: Rat liver mitochondria + Fe^{59} (768 c.p.m.) + ascorbic acid ($10^{-3} M$) + phosphate buffer pH 7.4 + protoporphyrin (up to saturation level at $\sim 67.5 \mu\text{g.}$).

DISCUSSION

Chicken red cells are capable of synthesizing protoporphyrin and also haem from suitable precursors. The preparation (CBZ) made from them by haemolyzing with benzene and discarding the insoluble material by centrifugation can not carry the synthesis beyond the stage of coproporphyrinogen. This system is completed, however, by the addition of rat liver mitochondria.

The chemical mechanism by which coproporphyrinogen is transformed into protoporphyrinogen or protoporphyrin is not yet known. The step involves the oxidative decarboxylation of two propionic acid side chains (β positions 2 and 4) and could be envisaged as proceeding first by dehydrogenation to form 2,4-diacrylyl deuteroporphyrin and then by decarboxylation of the latter. Alternatively, β -oxidation of the two propionic acid residues could lead to a porphyrin structure with two β -ketonic-acid side chains.

The latter, being inherently unstable, might then lose carbon dioxide. The porphyrin resulting would be 2,4-diacetyl deuteroporphyrin but this does not appear to be an intermediate in protoporphyrin biosynthesis.

It was hoped that a study of the part played by mitochondria in the coproporphyrinogen→protoporphyrin (ogen) transformation might throw light upon the chemical mechanism involved. The finding that a supernatant prepared from frozen and thawed mitochondria is as effective as the intact particles in completing the system, renders it unlikely that a fully functional electron transport chain is necessary. Perhaps the mitochondrial fraction supplies a particularly suited hydrogen acceptor; no artificial hydrogen acceptor has yet been found capable of completing the system. Since certain mitochondria, such as those of rat heart are ineffective, enzymological comparison of these with mitochondria from rat liver might shed further light upon this problem.

Iron incorporation into protoporphyrin also requires a system present in rat liver mitochondria and in this case a supernate prepared from them is inactive. From the work of others, it seems possible that the iron and possibly the protoporphyrin also, may be bound to protein before enzymic combination is effected.

SUMMARY

1. The preparation is described of an enzymic system (CBZ) from chick red cells which forms coproporphyrin but very little protoporphyrin from porphobilinogen.

Activity resides entirely in the non-ultrafilterable fraction. Some properties of the system have been investigated.

2. Addition to this system of rat liver mitochondria increases protoporphyrin formation at the expense of coproporphyrin. The supernate prepared by freezing and thawing rat liver mitochondria and centrifuging exhibits this activity as does also the extract from acetone-dried rat liver mitochondria. Activity is destroyed by heating.

3. Mitochondria from rat heart and

kidney do not increase protoporphyrin formation when added to CBZ and porphobilinogen. Mitochondria from rat spleen are only very feebly active in this respect.

4. Ferricyanide strongly inhibits porphyrin formation from porphobilinogen by CBZ and rat liver mitochondria; sodium fluoride is without effect but addition of iodoacetate increases protoporphyrin production.

o-phenanthroline appears to favour protoporphyrin formation but to have also a generally inhibitory effect upon the system. Cyanide tested aerobically and anaerobically had no significant effect upon uroporphyrin and coproporphyrin production by CBZ acting alone upon porphobilinogen.

5. Haem formation by the CBZ system has been investigated using either protoporphyrin as substrate or coproporphyrinogen plus rat liver mitochondria or porphobilinogen plus rat liver mitochondria. CBZ alone has only slight and somewhat variable activity in iron incorporation. This is increased by addition of ascorbic acid. Rat liver mitochondria have quite considerable activity which is also increased by addition of ascorbic acid but the most efficient system was constituted by CBZ plus rat liver mitochondria plus ascorbic acid.

6. The supernate from frozen and thawed and centrifuged rat liver mitochondria is almost entirely inactive as regards iron incorporation.

7. Anaerobic conditions favour haem formation from protoporphyrin and rat liver mitochondria; heating destroys the activity. Sodium fluoride is without effect.

REFERENCES

- (1) Rimington, C., *Rev. Pure and Applied Chem.* (Roy. Austral. Chem. Inst.), **8**, 129 (1958)
- (2) Reimann, F., *Schweitz. Zeit. Path. Bakt.*, **5**, 343 (1942); *ibid* **6**, 35 (1943)
- (3) London, I. M., Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **183**, 749 (1950)
- (4) Clark, P., and Walsh, R. J., *Nature, Lond.*, **184**, 1730 (1959)
- (5) Brunner, A., and Vallejo-Freire, A., *Exptl. Cell Research*, **10**, 55 (1956)
- (6) Braunstein, H., Fellinger, K., and Pakesch, F.

- Acta Haematol.*, **16**, 322 (1956)
- (7) Seno, S., Yoshizawa, Y., Nakamoto, T., and Kanda, S., *Folia Haematol.*, **2**, 269 (1958)
- (8) Sano, S., *Acta Haematol. Japon.* **21**, Suppl. **2**, 337 (1958)
- (9) Sano, S., *Acta Haematol. Japon.*, **18**, 625; *ibid* **18**, 631 (1955)
- (10) Sano, S., Inoue, S., Tanabe, Y., Sumiya, C., and Koike, S., *Science*, **129**, 275 (1959)
- (11) Shemin, D., and Russell, C. S., *J. Am. Chem. Soc.*, **75**, 4873 (1953)
- (12) Gibson, K. D., Laver, W. G., and Neuberger, A., *Biochem. J.*, **68**, 17 P (1938)
- (13) Laver, W. G., and Neuberger, A., *Biochem. J.*, **67**, 22 P. (1957)
- (14) Brown, E. G., *Biochem. J.*, **70**, 313 (1958)
- (15) Falk, J. E., Dresel, E. I. B., and Rimington, C., *Nature, Lond.*, **172**, 292 (1953)
- (16) Dresel, E. I. B., "Porphyrin Biosynthesis and Metabolism" Ciba Foundation Symposium, p. 72 (1955)
- (17) Rimington, C., and Booiij, H. L., *Biochem. J.*, **65**, 3 P (1957)
- (18) Dresel, E. I. B., and Falk, J. E., *Biochem. J.*, **57**, 476 (1954)
- (19) Rimington, C., and Sveinsson, S. L., *Scand. J. Clin. Lab. Invest.*, **2**, 209 (1950)
- (20) Lockwood, W. H., and Benson, A., *Biochem. J.*, **75**, 372 (1960)
- (21) Goldberg, A., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M., *Blood*, **11**, 821 (1956)
- (22) Kagawa, Y., Minakami, S., and Yoneyama, Y., *J. Biochem. (Japan)*, **46**, 771 (1959)
- (23) Schwartz, H. C., Cartwright, G. E., Smith, E. L., and Wintrobe, M. M., *Blood*, **14**, 486 (1959)
- (24) Grinstein, M., Bannerman, R. M., and Moore, C. V., *Blood* **14**, 476 (1959)
- (25) Lochhead, A. C., and Goldberg, A., *Biochem. J.*, **78**, 146 (1961)
- (26) Minakami, S., Yoneyama, Y., and Yoshikawa, H., *Biochim. et Biophys. Acta*, **28**, 447 (1958)
- (27) Minakami, S., *J. Biochem. (Japan)*, **45**, 833 (1958)
- (28) Nishida, G., and Labbe, R. F., *Biochim. et Biophys. Acta*, **31**, 519 (1959)
- (29) Faber, M., and Falbe-Hansen, I., *Nature, Lond.*, **184**, 1043 (1959)
- (30) Rabinovitz, M., and Olson, M. E., *Nature, Lond.*, **181**, 1665 (1958)
- (31) Eriksen, L., "Porphyrin Biosynthesis and Metabolism" Ciba Foundation Symposium, p. 185.
- (32) Bénard, H., Gajdos, A., and Gajdos-Török, M., *Compt. rend. Soc. Biol.*, **147**, 92 (1953)

γ -Hydroxyarginine, a New Guanidino Compound from a Sea-cucumber

III. Actions of Arginase and Arginine Decarboxylase

By YOSHIMASA FUJITA

(From the Department of Chemistry, Faculty of Science, Kyushu University, Fukuoka)

(Received for publication, February 10, 1961)

The isolation and identification of γ -hydroxy-L-arginine from a sea-cucumber, *Polysiphonia rufescens* (1), and the determination of its stereoconfiguration (2) were reported previously by the present author. In these studies it was found that the amino acid was susceptible to arginase and L-amino acid oxidase and thereby converted to γ -hydroxy-L-ornithine and γ -guanidino- β -hydroxybutyric acid, respectively. Recently, the occurrence of γ -hydroxyarginine in a sea-anemone, *Anthopleura japonica*, was found by Makisumi in this laboratory, who isolated simultaneously from this animal γ -guanidino- β -hydroxybutyric acid and γ -hydroxyagmatine corresponding to the oxidation and decarboxylation products of γ -hydroxyarginine, respectively (3). To identify γ -hydroxyagmatine, a new guanidino compound, Makisumi synthesized it enzymically by using arginine decarboxylase from *Escherichia coli* 7020 and No. 1 (4).

In all these enzymatic reactions, it was observed that the rates of conversion of γ -hydroxyarginine to each of the reaction products were considerably low in comparison with those of arginine. It is obvious that the steric hindrance caused by the presence of a hydroxy group at γ -position of this compound is responsible for the decrease in the rates. Accordingly, it is interesting to measure quantitatively the effect of the presence of γ -hydroxy group on the velocity of enzymatic reaction.

In the present experiment, the rates of reaction of γ -hydroxyarginine catalyzed by arginase and arginine decarboxylase were determined and compared with those of arginine

catalyzed by the same enzymes, and the Michaelis constants for γ -hydroxyarginine in these enzymatic reactions were also calculated.

MATERIALS

Substrates—L-Arginine monohydrochloride is a commercial product. γ -Hydroxy-L-arginine monohydrochloride is a preparation isolated from the sea-cucumber. The stereoconfiguration of its γ -hydroxy group is *erythro*-form; m. p. 190–191°C (decomp.), $[\alpha]_D^{25} + 5.4^\circ$ ($c=2$, in 5 *N* hydrochloric acid).

Arginase—A partially purified beef liver arginase preparation obtained as a solution in the Step D according to Greenberg (5) was used without dilution in the incubation with γ -hydroxyarginine, while in the case of arginine the arginase solution was diluted with water ten times.

Urease—For determination of urea a crude urease preparation purchased from Ishizu Pharmaceutical Co. Ltd. was used without further purification.

Arginine Decarboxylase—Though it is well known that *Escherichia coli* 7020 is specific for the decarboxylation of arginine alone (6), it was tested for the decarboxylation of γ -hydroxyarginine as Hagiwara (7) and Suzuki *et al.* (8) found recently that the organism also decarboxylated L-canavanine which is a structural analogue of arginine. After preparing an acetone powder of the organism according to the method of Gale (9), it was suspended in 0.2 *M* phosphate-citrate buffer pH 5.2 (10 mg./ml.) to obtain an arginine decarboxylase solution.

EXPERIMENTALS AND RESULTS

Arginase

pH Optimum of Arginase for the Hydrolysis of γ -Hydroxyarginine—An assay mixture containing each 0.5 ml. of 0.1 *M* γ -hydroxyarginine, 0.01 *M* manganous sulfate, 0.4 *M* Michaelis' veronal buffer (pH range from 7 to 9.6)

and the arginase solution was incubated at 30°C for 10 minutes. For the suppression of arginase action, the mixture was added with 0.5 ml. of 1.2 *N* sulfuric acid and heated in a boiling water bath for 5 minutes, then cooled to room temperature. For determination of urea to be produced, an urease method was applied as follows. To the resulting mixture 0.5 ml. of 1.6 *N* phosphate buffer was added (the pH of the mixture should be 6.8, otherwise, it should be corrected by adding an adequate amount of 2 *N* sodium hydroxide or 2 *N* sulfuric acid). After further addition of 0.5 ml. of 0.05 *M* EDTA and the same volume of an urease solution (10 mg. of the urease/ml. of water) to the mixture, it was incubated again at 30°C for 60 minutes. Then ammonia in an aliquot of the incubation mixture was analyzed by using the Conway microdiffusion method. The pH optimum of arginase for the hydrolysis of γ -hydroxyarginine was approximately 9.2 under these experimental conditions (Fig. 1). Consequently, as there was no appreciable difference between the pH optima of arginase action on arginine and on γ -hydroxyarginine, subsequent experiments were carried out at this pH.

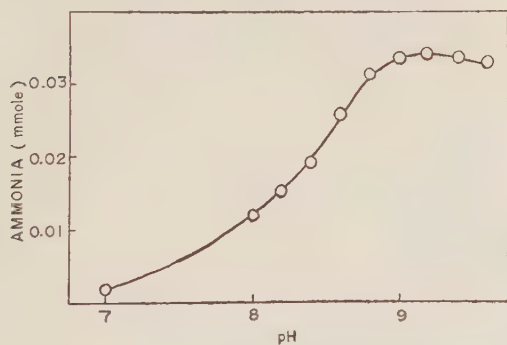


FIG. 1. pH-Activity curve of arginase for γ -hydroxyarginine. Reaction mixture: 0.5 ml., 0.1 *M* γ -hydroxyarginine; 0.5 ml., 0.01 *M* manganous sulfate; 0.5 ml., 0.4 *M* veronal buffer; 0.5 ml., arginase solution. Temperature, 30°C. Time, 10 minutes.

Determination of the Hydrolysis Rates—The rates of hydrolysis by arginase of arginine and γ -hydroxyarginine having several substrate concentrations at pH 9.2 were determined

in a similar manner to the case of the determination of pH optimum (Fig. 2). The apparent velocity constants for the hydrolysis

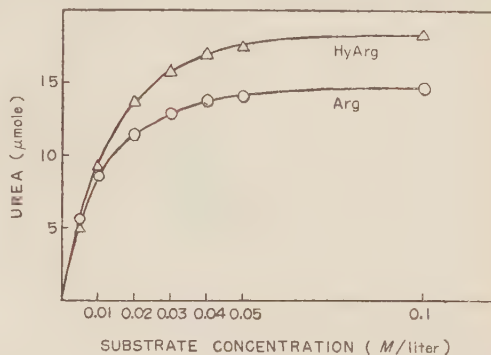


FIG. 2. Effect of substrate concentration on arginase activity. Reaction mixture: 0.5 ml., substrate; 0.5 ml., 0.01 *M* manganous sulfate; 0.5 ml., 0.4 *M* veronal buffer pH 9.2; 0.5 ml., arginase solution (Ten times diluted solution was used in the case of ARG.). Temperature, 30°C. Time, 10 minutes.

of the two substrates and the ratio of the two constants at the same substrate concentration were calculated. The results are summarized in Table I. The rate of hydrolysis of γ -hydroxyarginine by arginase was approximately 11 per cent that of arginine with the substrate concentration from 0.005 to 0.05 *M*.

TABLE I
Apparent Velocity Constants of Hydrolysis of Arginine and γ -Hydroxyarginine and Their Ratios

Substrate Concentration (10^{-2} <i>M</i>)	$k_A^{1)}$	$k_{HA}^{2)}$	k_{HA}/k_A %	Average Ratio %
0.5	0.0810	0.00685	8.5	11
1.0	0.0575	0.00516	9	
2.0	0.0336	0.00375	11	
3.0	0.0243	0.00306	13	
4.0	0.0190	0.00239	13	
5.0	0.0150	0.00193	12	

1) Velocity constant for arginine

2) Velocity constant for γ -hydroxyarginine

The Michaelis Constant—By using the velocity constants obtained above, the Michaelis

constants for these substrates were calculated according to the Lineweaver-Burk's equation (Fig. 3). The constant for γ -hydroxyarginine was $1.6 \times 10^{-2} M$, while for arginine was $5 \times 10^{-3} M$.

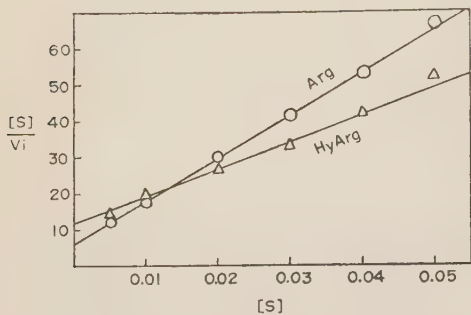


FIG. 3. The plots of initial substrate concentration $[S]$ divided by initial velocity V_i versus $[S]$ for the hydrolysis of the substrates by arginase at $30^\circ C$ and pH 9.2.

Arginine Decarboxylase

Determination of the Decarboxylation Rates—
The decarboxylations of arginine and γ -hydroxyarginine were carried out in the Warburg manometers filled with air at $30^\circ C$. A mixture consisting of 1.0 ml. of the freshly prepared suspension of acetone powder of *E. coli* and of 0.5 ml. of 0.2 M phosphate-citrate buffer (pH 5.2) was placed in the main compartment, and 1.0 ml. of 0.0025 to 0.0125 M substrate solution was placed in the side arm.

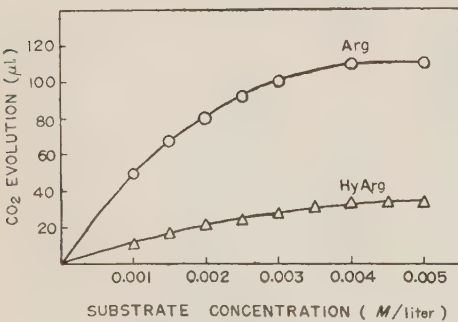


FIG. 4. Effect of substrate concentration on arginine decarboxylase activity. Contents of vessel: 1.0 ml., enzyme solution; 0.5 ml., 0.2 M phosphate-citrate buffer pH 5.2; 1.0 ml., substrate solution. Temperature, $30^\circ C$. Time, 5 minutes.

The evolution of carbon dioxide in 5 minutes was measured after equilibration of the temperature (Fig. 4). The apparent velocity constants for decarboxylation of the two substrates and the ratio between the two constants at the same substrate concentration were calculated. The results are summarized in Table II. The rate of decarboxylation of γ -hydroxyarginine by arginine decarboxylase from *E. coli* 7020 was approximately 16.5 per cent that of arginine with the substrate concentration from 0.0015 to 0.003 M.

TABLE II

Apparent Velocity Constants of Decarboxylation of Arginine and γ -Hydroxyarginine and Their Ratios

Substrate Concentration ($10^{-3} M$)	$k_A^{1)}$	$k_{HA}^{2)}$	k_{HA}/k_A %	Average Ratio %
1.0	0.399	0.0375	9.4	16.5
1.5	0.321	0.0422	13.1	
2.0	0.250	0.0390	15.5	
2.5	0.207	0.0366	17.7	
3.0	0.178	0.0351	19.7	
4.0	0.134	0.0327	24.4	
5.0	0.099	0.0260	26.3	

- 1) Velocity constant for arginine
- 2) Velocity constant for γ -hydroxyarginine

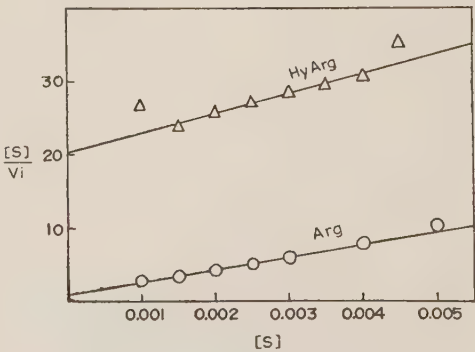


FIG. 5. The plots of initial substrate concentration $[S]$ divided by initial velocity V_i versus $[S]$ for the decarboxylation of the substrates by arginine decarboxylase at $30^\circ C$ and pH 5.2.

The Michaelis Constant—The constants for the two substrates were calculated in a similar manner to the case of hydrolysis by arginase

and the following values were obtained; $7.6 \times 10^{-3}M$ for γ -hydroxyarginine and $4.1 \times 10^{-4}M$ for arginine (Fig. 5). The value for arginine was in good accordance with that reported by Gale (10).

SUMMARY

The reaction rates of hydrolysis and decarboxylation of γ -hydroxyarginine catalyzed by arginase and arginine decarboxylase were determined and compared with those of arginine. The apparent reaction velocity of hydrolysis of γ -hydroxyarginine was about 11 per cent of that of arginine, and that of decarboxylation was about 16.5 per cent. The Michaelis constants of arginase and arginine decarboxylase for γ -hydroxyarginine were also calculated and the following data were obtained; $1.6 \times 10^{-2}M$ in the arginase reaction and

$7.6 \times 10^{-3}M$ in the decarboxylase reaction.

The author wishes to express his cordial thanks to Prof. S. Shibuya for his guidance and encouragement. His thanks are also due to the Ministry of Education for financial support of this research.

REFERENCES

- (1) Fujita, Y., *Bull. Chem. Soc. Japan*, **32**, 439 (1959)
- (2) Fujita, Y., *Bull. Chem. Soc. Japan*, **33**, 1379 (1960)
- (3) Makisumi, S., *J. Biochem.*, **49**, 284 (1961)
- (4) Makisumi, S., *J. Biochem.*, **49**, 292 (1961)
- (5) Greenberg, D. M., "The Enzymes," Academic Press Inc., **I**, 905 (1951)
- (6) Gale, E. F., "Methods of Biochemical Analysis," Interscience Publishers, Inc., **IV**, 299 (1957)
- (7) Hagihara, F., *J. Pharm. Soc. Japan*, **76**, 613 (1956)
- (8) Suzuki, T., Muraoka, S., and Konobu, K., *J. Pharm. Soc. Japan*, **74**, 540 (1954)
- (9) Gale, E. F., *Biochem. J.*, **39**, 52 (1945)
- (10) Gale, E. F., *Biochem. J.*, **34**, 392, 846 (1940)

On the Mechanism of Transpyrophosphorylation in the Biosynthesis of Thiamine Diphosphate

By YOSHITO KAZIRO, RYO TANAKA, YOSHITAKE MANO
and NORIO SHIMAZONO

(From Department of Biochemistry, Faculty of Medicine,
University of Tokyo, Tokyo)

(Received for publication, February 11, 1961)

The enzyme thiaminokinase catalyzes the phosphorylation of thiamine by ATP* and the product of the reaction was identified as TDP, *i.e.* cocarboxylase. Concerning the reaction mechanism Weil-Malherbe (1) suggested by the use of a crude preparation obtained from brewer's yeast that it is a one-step transpyrophosphorylation from ATP to thiamine, rather than a stepwise transphosphorylation, because thiamine is a more effective substrate than TMP. Leuthardt and Nielsen (2) supported subsequently this hypothesis from a similar observation with a partially purified enzyme from rat liver. Although this view has been generally accepted among other possibilities (3-5) and has also been cited by many authors (6-8), no conclusive evidence had so far been obtained until the present work was undertaken.

The purpose of the present work is to demonstrate the transfer of an intact pyrophosphate group from ATP to thiamine by the use of a terminally labeled ATP-P³², and at the same time, to distinguish between the two possibilities shown in the following formulae.



While the present work was in progress,

* Abbreviations used: ATP or ARPPP, adenosine triphosphate; ADP, adenosine diphosphate; AMP or ARP, adenosine monophosphate; R-5-P, ribose-5-phosphate; TDP or TPP, thiamine diphosphate; TMP, thiamine monophosphate; T, thiamine; Th, 4-methyl-5-β-hydroxyethylthiazole; OMP, 2-methyl-4-amino-5-hydroxymethylpyrimidine; and Tris, tris(hydroxymethyl)aminomethane.

Khorana *et al.* (9) succeeded in demonstrating that pyrophosphate group is transferred by the similar mechanism as shown in equation (I), with the enzyme phosphoribose pyrophosphokinase, one of the two enzymes which have been believed to catalyze pyrophosphorylation (10). More recently, Greiling (11) reported on the phosphorylation mechanism of thiamine with yeast enzyme and stated that the activity ratio of β- to α-P atom of TDP,** which was synthesized enzymatically from ATP-γ-P³², was 3 to 1. From this value, the randomization of the labeling pattern of ATP by adenylate kinase or other contaminating enzymes was suspected, although the activity ratio of the ATP used was not specified in his report. Forsander (12)*** reported a similar work on yeast thiaminokinase a few years ago, and concluded that the P³² of ATP-γ-P³² was transferred to thiamine as the β-P of TDP from the findings of the radioactivity on paper chromatogram. Although his results gave a strong support to the transfer of pyrophosphate group, the conclusion seemed not to be quite definitive since TDP and TMP were not separated completely from other substances and also the data were by no means quantitative.

In order to provide a definitive evidence for this mechanism, it is necessary to carry

** Each P atom in ATP and TDP is distinguished as α, β and γ, and α and β, respectively; AR-P(α)-P(β)-P(γ) and T-P(α)-P(β).

*** The detail of Forsander's work was not available to the authors before the present work was finished.

out the reaction of ATP- γ -P³² of high specific activity and a purified enzyme which are completely free of other interfering enzymes such as adenylate kinase.

In this paper are described the studies on the mechanism of transpyrophosphorylation with the purified thiaminokinase, using ATP- γ -P³² which was synthesized enzymatically from carbamyl phosphate-P³². Purification, properties, and nucleotide specificity of this enzyme were described in the preceding papers (13, 14). A preliminary communication of the present experiments has also been published elsewhere (15).

EXPERIMENTAL

Materials—ATP labeled only on the terminal phosphate was synthesized from labeled carbamyl phosphate by reverse reaction of carbamate kinase. The details of this method have been given elsewhere (16, 17). The specific activity of ATP used for this experiment was 3.86×10^6 c.p.m. per μ mole and the ratio of specific activity of γ - to β -P atom was 11.5 to 1. Thiamine, TMP and TDP were kindly supplied by Takeda Chemical Industries, Ltd., Japan. TDP was further purified by ion exchanger chromatography as described below.

Enzymes—Thiaminokinase was purified about 100 fold from autolysate of baker's yeast as previously described (13). The purified enzyme showed no adenylate kinase activity when assayed by pyruvic kinase-lactic dehydrogenase system with ATP plus AMP as substrate (13). With this purified enzyme, ADP could not substitute for ATP as phosphate donor and TMP was not phosphorylated (13). Pyruvic carboxylase was also prepared from baker's yeast autolysate (18) and resolved to apoenzyme by alkaline ammonium sulfate treatment according to the method of Green *et al.* (19).

Methods for Isolation, Identification, and Determination of Thiamine Phosphates—The reaction products were isolated by ion exchanger chromatography on Dowex-1 column (chloride form, X-4, 200 to 400 mesh). The elution was made stepwise based on the HCl-NaCl system according to Cohn and Carter (20). Thiamine phosphates were separated by successive elution from the column with 0.001 *N* HCl (for TMP) and 0.002 *N* HCl (for TDP). Each peak was identified spectrophotometrically and, after lyophilization, by paper chromatography using a solvent system of Siliprandi and Siliprandi (21). The spots on paper chromatogram were located by ultra-

violet absorption, thiochrome fluorescence, and radioactivity. The *R_f* values were 0.31, 0.15 and 0.07 for thiamine, TMP and TDP, respectively.

TMP and TDP were determined after elution from Dowex-1 column by their absorbance at 260 m μ (pH 3.0). TDP was also measured by its coenzyme activity after recombination with apocarboxylase (22).

RESULTS

Reaction Conditions—The reaction mixture composed of 3 mmoles of Tris buffer, pH 8.6; 150 μ moles of MnSO₄; 150 μ moles of thiamine; 15 μ moles of ATP- γ -P³² (specific activity, 3.86×10^6 c.p.m. per μ mole; γ/β , 11.5/1); approximately 6 mg. of purified enzyme and water in a final volume of 150 ml. The mixture was incubated at 30°C for 45 minutes, after which time the reaction was stopped by boiling for 2 minutes at 100°C. After centrifugation a suitable aliquot was taken from the supernatant solution and TDP formed was measured manometrically using apocarboxylase prepared from baker's yeast. About 0.31 μ mole of TDP was synthesized under these conditions. The supernatant was adjusted to pH 7.4, diluted about 6 fold, and loaded on a Dowex-1 column (chloride form, X-4, 1.1 \times 8 cm.). Ten μ moles of nonlabeled TDP was added as a carrier.

Separation of the Reaction Products—The column chromatography was developed as described in Experimental Section. Each peak was identified from its ultraviolet spectrum, thiochrome test, radioactivity, and coenzyme activity. Further confirmation was obtained from paper chromatography after lyophilization. The results of the column chromatography are shown in Fig. 1. The peaks B, C and D were identified as TMP, TDP and AMP, respectively. Peak A seemed to be of protein nature and was not further studied because it contained very little radioactivity. Peak B was identified as TMP, which was presumably formed nonenzymatically from TDP. As is clear from Fig. 1, the main product of the reaction was TDP. About 0.7×10^6 c.p.m. of TDP was recovered from the column.

Preliminary analysis of the labelling

pattern of TDP was performed at this stage as follows: 0.5 ml. aliquot which contained about 6,600 c.p.m. was hydrolyzed with 0.25 ml. of 3 *N* H₂SO₄ s at 100°C for 45 minute. Under these conditions, only terminal phosphate of TDP was liberated. After cooling

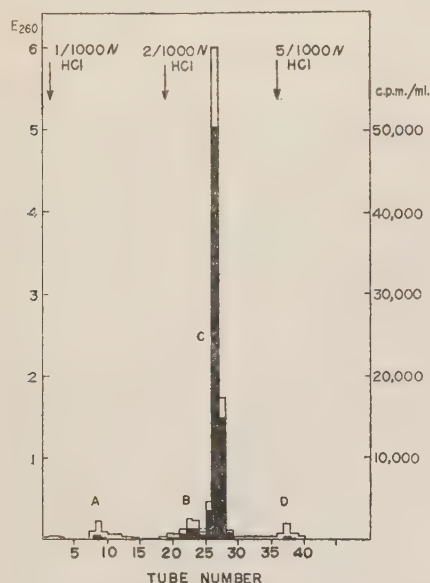


FIG. 1. Column chromatography of the reaction products.

After deproteinization, the reaction products were adsorbed on Dowex-1 column (chloride form, X-4, 1.1×8.0 cm.) with 10 μ moles of nonlabeled TDP. The chromatogram was developed as described in the text. Flow rate, 2 ml. per minute; fraction volume, 10 ml. □, Absorbancy at 260 $m\mu$; ■, radioactivity in c.p.m./ml. of the fraction.

in an ice-bath, 0.75 ml. of water and 0.5 ml. of 2% ammonium molybdate were added. Inorganic phosphate derived from β -P atom of TDP was extracted three times with isobutanol. The radioactivity of the aqueous layer (which corresponded to α -phosphate in TDP) was 1,470 counts per 3 minutes and that of the organic layer (which corresponded to β -phosphate in TDP) was 18,110 counts per 3 minutes.

Distribution of P^{32} on α - and β -P atoms of TDP—The radioactive TDP was purified further by rechromatography on a small column (4×0.6 cm.) of Dowex-1 with the

hydrochloric acid system and its specific activity was determined as 121.4×10^3 c.p.m. per μ mole based on the absorbance at 260 $m\mu$. This TDP was hydrolyzed with *N* hydrochloric acid at 100°C for 30 minutes to split it in a way that enables differentiation of the phosphates of the pyrophosphoryl group. After hydrochloric acid was removed by lyophilization, resulting TMP and inorganic phosphate were separated again by column chromatography as above and the specific activity of TMP was found to be 9.7×10^3 c.p.m. per μ mole.

From these results the activity ratio of β - to α -P atom of TDP produced was calculated to be about 11.5/1, which was equal to that of γ - to β -P atom of ATP used for the experiments. The purity of TDP and TMP thus isolated was checked by paper chromatography and both of them were detected as single spots by ultraviolet absorption and thiochrome fluorescence.

DISCUSSION

The results presented here establish definitely the transfer of an intact pyrophosphoryl group from ATP to thiamine by the mechanism shown in equation (I). This mechanism seems to be essentially same as

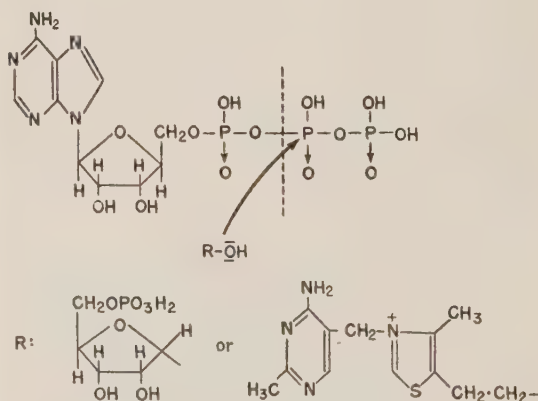


FIG. 2. Mechanism of transpyrophosphorylation.

that of the phosphoribose pyrophosphokinase. In the latter case Kornberg (6) suggested that the enzyme catalyzes a nucleophilic attack of oxygen atom in R-5-P on the

middle phosphorus atom of ATP with the displacement of AMP (Fig. 2).

From the experimental results so far available, however, it is still impossible, in either of these two cases, to distinguish between the following two possibilities. First, the enzyme catalyzes, after combination with both substrates, the nucleophilic attack of oxygen atom in hydroxyl group on the middle phosphorus in ATP. The second possibility is that a negative group on enzyme protein first makes a nucleophilic attack to form PP-enzyme complex, with subsequent displacement of enzyme by acceptor molecule, thus in the case of thiaminokinase:



The isotope exchange between AMP-C¹⁴ and ATP in the absence of thiamine will provide information on this point. It is also supposed in the foregoing argument, on the analogy of many other transphosphorylating enzymes, that P-O bond will be cleaved as indicated in Fig. 2. However, direct evidence must await O¹⁸ experiments.

Other two reactions have so far been reported to involve the pyrophosphate derivatives as activated form. One is dimethylallyl-pyrophosphate which participates in the biosynthesis of cholesterol. This compound is now known to be derived from mevalonic pyrophosphate which is formed by two-step phosphorylation and not by one-step pyrophosphorylation (25). The other example is the case of OMP-pyrophosphate which condenses with Th-P to form thiamine monophosphate (24-30). From the recent report of Kawasaki *et al.* (27), it seems likely that the synthesis of OMP-pyrophosphate also takes place by two-step phosphorylation rather than one-step pyrophosphorylation.

Recently, the pathway of biosynthesis of TDP from OMP and Th was studied by several groups of investigators. Nose *et al.* (26, 28) and Leder (25) supposed that OMP-PP will react with Th-P to form TMP which is then phosphorylated by ATP to form TDP. They proposed, therefore, a pathway which exclude free thiamine as an intermediate in

TDP biosynthesis. However, an objection was raised by the present authors against the above hypothesis and an alternative possibility was suggested in which TMP is once hydrolyzed to free thiamine and subsequently pyrophosphorylated to form TDP. The latter hypothesis is based on the observation that even with the crude extract of baker's yeast, free thiamine is a better substrate than either TMP or OMP plus Th*. This view was also presented independently by Camiener and Brown (29, 30). It seems more reasonable because of the wide-spread distribution of the thiaminokinase in various microorganisms which can synthesize thiamine from low-molecular precursors as well as in the higher animals which require dietary supply of thiamine.

Since the mechanism of the reaction is now clearly established, the name thiamine pyrophosphokinase is proposed for this enzyme**.

SUMMARY

Terminally labeled ATP-P³² was incubated with thiamine in the presence of a thiaminokinase purified from baker's yeast. The analysis of TDP formed showed that the ratio of radioactivity of β - to α -P atom in TDP was equal to that of γ - to β -P atom in ATP used for the experiments. The mechanism of this enzyme reaction was thus demonstrated to involve a transfer of an intact pyrophosphoryl group from ATP to thiamine as indicated by Equation (I). The name thiamine pyrophosphokinase was proposed for this enzyme.

REFERENCES

- (1) Weil-Malherbe, H., *Biochem. J.*, **33**, 1997 (1939)
- (2) Leuthardt, F., and Nielsen, H., *Helv. Chim. Acta*, **35**, 1196 (1952)
- (3) Nguyen-Van Thoai and Chevillard, L., *Compt. rend.*, **232**, 1444 (1951)
- (4) Nguyen-Van Thoai, Roche, J., and Sfez, M.,

* Unpublished observation

** This name was suggested also by Camiener and Brown (30) independently of the present authors during the preparation of this paper.

- Compt. rend.*, **234**, 891 (1952); **236**, 243 (1953)
- (5) Steyn-Parvé, E. P., *Biochim. et Biophys. Acta*, **8**, 310 (1952)
- (6) Kornberg, A., *Advances in Enzymology*, **18**, 191 (1957)
- (7) Dixon, H., and Webb, E. C., in "Enzymes," Longmans, Green and Co., London, New York, Toronto, p. 214, 235 (1955)
- (8) Mano, Y., *J. Biochem.*, **47**, 24 (1961)
- (9) Khorana, H. G., Fernandes, J. F., and Kornberg, A., *J. Biol. Chem.*, **230**, 941 (1958)
- (10) Kornberg, A., Lieberman, I., and Simms, E. S., *J. Biol. Chem.*, **215**, 389 (1955)
- (11) Greiling, H., 'Zusammenfassungen, IV. Internationaler Kongress der Biochemie, Wien,' p. 46 (1958)
- (12) Forsander, O., *Soc. Sci. Fennica, Commentationes Phys.-Math.*, **XIX**, 2 (1956)
- (13) Kaziro, Y., *J. Biochem.*, **46**, 1523 (1959)
- (14) Kaziro, Y., *J. Biochem.*, **46**, 1587 (1959)
- (15) Shimazono, N., Mano, Y., Tanaka, R., and Kaziro, Y., *J. Biochem.*, **46**, 959 (1959)
- (16) Tanaka, R., Mano, Y., and Shimazono, N., *Biochim. et Biophys. Acta*, **36**, 262 (1959)
- (17) Tanaka, R., *J. Biochem.*, **47**, 207 (1960)
- (18) Kaziro, Y., *J. Biochem.*, **44**, 827 (1957)
- (19) Green, D. E., Herbert, D., and Subrahmanyam, V., *J. Biol. Chem.*, **138**, 327 (1941)
- (20) Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72**, 4273 (1950)
- (21) Siliprandi, D., and Siliprandi, N., *Biochim. et Biophys. Acta*, **14**, 52 (1954)
- (22) Aoshima, Y., *J. Japan. Biochem. Soc.*, (in Japanese), **29**, 861 (1958)
- (23) Block, K., Chaykin, S., Phillips, H., and de Waard, A., *J. Biol. Chem.*, **234**, 2595 (1959)
- (24) Harris D. L., and Yavit, J., *Federation. Proc.*, **16**, 192 (1957)
- (25) Leder, I. G., *Biochem. Biophys. Research Communications*, **1**, 63 (1959)
- (26) Nose, Y., Ueda, K., and Kawasaki, T., *Biochim. et Biophys. Acta*, **34**, 277 (1959)
- (27) Kawasaki, T., Fujita, T., Iwashima, A., Ueda, K., and Nose, Y., 'Abstracts of 12th Symposium on Enzyme Chemistry,' Sendai, Japan (in Japanese) p. 68 (1960)
- (28) Nose, Y., Ueda, K., and Kawasaki, T., 'Abstracts of 11th Symposium on Enzyme Chemistry,' Kyoto, Japan (in Japanese), p. 163 (1959)
- (29) Camiener, G. W., and Brown, G. M., *J. Am. Chem. Soc.*, **81**, 3800 (1959)
- (30) Camiener, G. W., and Brown, G. M., *J. Biol. Chem.*, **235**, 2411 (1960)

Chemical Studies on Cellular Components of *Hemophilus pertussis*

II. Isolation of Toxic Lipopolysaccharide

By MASAYASU KITAGAWA,⁺ HIDEBUMI HAZAMA,⁺ MASAHARU MOTOMURA,⁺
YUICHI YAMAMURA,⁺ KEI SAWADA⁺⁺ and JUNJI NAGAI⁺⁺

(From ⁺the Department of Biochemistry, Faculty of Medicine, Kyushu University, Fukuoka
and ⁺⁺Central Clinical Laboratory, Faculty of Medicine, Kyushu University, Fukuoka)

(Received for publication, February 20, 1961)

The occurrence of biological active lipopolysaccharides in many species of Gram-negative bacteria, has been reported by many investigators. They have been referred to as endotoxins or Boivin antigen. The similar substances have been demonstrated in *Hemophilus pertussis* (1-6). Cruickshank and Freeman (1) isolated a material containing polysaccharides from the tryptic digest of *Hemophilus pertussis* phase I. Recently, MacLennan (2, 3) reported the chemical and biological properties of specific lipopolysaccharides obtained from the same organisms. These preparations, however, have never been lethal for laboratory animals, though they had some biological activities such as immunizing potency (1) and pyrogenicity (3).

It has been known that pertussal vaccine was sometimes toxic for animals. Since the well-known thermolabile necrotoxin characterized as protein,* has been inactivated in the course of preparation of vaccine, there must be other stable toxic substances in this organism. From this point of view, the lipopolysaccharide substance, which may be responsible for a part of toxicity in vaccine, was prepared from *Hemophilus pertussis*. In the present study, the isolation method and the toxicity of the lipopolysaccharide have been investigated.

MATERIALS AND METHODS

Bacteria—The *Hemophilus pertussis* of Sakurayashiki strain in phase I were supplied by Takeda Pharmaceutical Industries, Ltd. Stock cultures grown on

Bordet-Gengou agar medium were subcultured in Roux bottle on semisynthetic culture medium containing casamino acid and charcoal. After incubation at 38°C for 48 hours, the organisms were harvested using 0.15 M sodium chloride solution by centrifugation. The paste of cells was stored in frozen state until needed.

Total Nitrogen—The micro-Kjeldahl method was used, with distillation apparatus of Parnas.

Phosphorus—Determinations were made by the method described by Barton (7).

Paper Chromatography—Sugars: Samples were hydrolysed in sealed ampoules at 100°C with NH_2SO_4 for 5 hours. For application to paper chromatograms, salt free preparations were made from H_2SO_4 hydrolysates by adding saturated $\text{Ba}(\text{OH})_2$ and adjusting pH to 4.7. The supernatant after centrifugation was evaporated *in vacuo* to dryness. Another desalting was carried out by treatment of hydrolysate with Dowex 1X2 (CO_3^{--} form) and IR 120 (H^+ form). The development solvents were pyridine-acetic ethylester-water-acetic acid (5:5:3:1) and butanol-acetic acid-water (4:1:5). Paper (Toyo Roshi No. 51) were sprayed with aniline phthalate (8), ammoniacal silver nitrate or ninhydrin solution in order to detect reducing sugars or hexosamine.

Amino Acid: Samples were hydrolysed in sealed ampoules at 110°C 6 N HCl for 24 hours. Acid was removed from HCl hydrolysates by repeated evaporation on water bath. Amino acids were adsorbed to IR 120 (H^+ form) and eluted with 5 N ammoniacal water. Eluate was dried up by repeated evaporation on water bath and applied to two dimensional paper chromatograms. Development solvents were butanol-acetic acid-water (4:1:5) and butanol-methylethyl ketone-17 N ammoniacal water-water (5:3:1:1).

Ultracentrifugation—Preparative ultracentrifuge runs were made in Hitachi preparative ultracentrifuge type 40 P at the low temperature. Figures quoted for the force in g refer to the force exerted at the centres of

* K. Onoue *et al.* unpublished

the tubes.

Toxicity—The test samples were dissolved in buffered saline (*M*/30 phosphate buffer, pH 8.3) and injected intraperitoneally into each groups of five mice. The death and the decrease of the body weight were observed during three days after injection. The mice used were ddN strain.

Immunizing Potency—The immunity against intraperitoneal challenge was tested in mice previously immunized with samples. This was carried out by Dr. H. Takehara, Takeda Pharmaceutical Industries, Ltd.

RESULTS

Extaction and Purification—The paste of bacterial cells was ground in mortar, with a half weight of glass powder and four times volume of 0.15 *M* sodium chloride and centrifuged at 20,000 r.p.m.

The cell debris from which was removed the most part of protein containing the necrotoxin by centrifugation, was used as starting material for the extraction of lipopolysaccharide. Thirty grams of cell debris (containing 10 g. glass powder) were added with 800 ml. of distilled water and homogenized in Waring blender.

The homogenous suspension was cooled to 2°C and added with 80 ml. of 90% phenol under the vigorous stirring and left overnight at this temperature under the furthur stirring.

The mixture was centrifuged at 3,000 r.p.m. and precipitate was discarded.

The milky extract was dialysed against the running water for 48 hours at room temperature and against the distilled water at 2°C to 4°C for 24 hours and centrifuged at 6,000 r.p.m., discarding the precipitate.

The milky spernatant was added with saturated sodium acetate, adjusting the reaction to be slightly alkaline with phenol red and cooled to 0°–2°C. Then, four times volume of 95% ethanol was added under the constant stirring.

After standing overnight at the cold room, the resulting precipitate was collected by centrifugation at 3,000 r.p.m., The precipitation by ethanol was repeated three times. The precipitate (designated as P_{alc}) was dissolved in 200 ml. of distilled water and

centrifuged at 15,000×*g* for one hour. The supernatant separated from the precipitate (designated as P₁₅), was centrifuged at 40,000×*g* for one hour. Then, the supernatant separated from precipitate (designated as P₄₀), was centrifuged at 105,000×*g* for one hour. The resulting precipitate was washed by repeated centrifugations at 105,000×*g* twice from aqueous solution. The last precipitate was designated as P₁₀₅ and the combined supernatant as S₁₀₅. Each fraction of P_{alc}, P₁₅, P₄₀, P₁₀₅ and S₁₀₅ was frozen-dried. Their yield are shown in Table I.

TABLE I

The Contents of Nitrogen and Phosphorus of Each Fraction Prepared from H. pertussis

Fraction	Yield	Nitrogen	Phosphorus
	mg.	%	%
P _{alc}		9.04	2.04
P ₁₅	420	12.75	0.65
P ₄₀	475	9.98	0.45
S ₁₀₅	400	8.65	4.31
P ₁₀₅	78	5.13	1.45

P_{alc}: Ethanol precipitate, S₁₀₅: Supernatant at 105,000×*g*, P₁₅, P₄₀, P₁₀₅: Precipitates at 15,000×*g*, 40,000×*g*, 105,000×*g*.

Hydrolysis of P_{alc} with *N* H₂SO₄ for 5 hours at 100°C, gave six spots on paper chromatograms, reacting with anilin-phthalate and ammoniacal silver nitrate. Three spots corresponded to galactose, glucose and xylose respectively and one spot reacting with ninhydrin was hexosamine and two spots, showing the slowest and the fastest migration on chromatograms, were unidentified.

With ninhydrin, ten spots were obtained from hydrolysate of 6 *N* HCl for 20 hours at 110°C on paper chromatograms.

These were identified as glutamic acid, aspartic acid, leucine, valine, glycine, proline, alanine, threonine, serine and histidine respectively but colamine and necrosamine were not found.

As seen in Fig. 1, ultraviolet-absorption spectrum of the P₁₀₅ fraction did not show any peak in the range of 240 mμ to 300 mμ of

wave length, suggesting that this fraction was free of nucleic acid, though the P_{alc} fraction showed the considerable contaminants of

nucleic acid. The determined values of C, H, N, P and ashes of the P₁₀₅ fraction were as follows:

C: 45.16 per cent, H: 8.20 per cent, N: 5.13 per cent, P: 1.45 per cent, ash: 7.37 per cent.

The detailed chemical properties of P₁₀₅ fraction will be reported in proceeding paper.

Toxicity—The various amounts of each fraction were injected intraperitoneally into

TABLE II

The Lethality of each Fraction Prepared from H. pertussis

Fraction	MLD
	mg.
P _{alc}	6
P ₁₅	15
P ₄₀	7
S ₁₀₅	20
P ₁₀₅	0.7

P_{alc}, P₁₅, P₄₀, P₁₀₅, and S₁₀₅ are similar in Table I

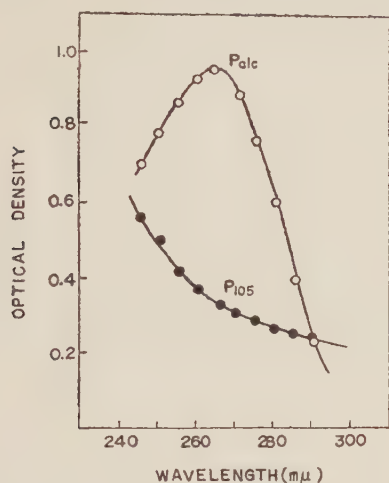


FIG. 1. Ultraviolet absorption spectra.

—○— P_{alc} fraction 0.05 mg./ml.

—●— P₁₀₅ fraction 0.05 mg./ml.

TABLE III

The Decrease of Body Weight by P_{alc} Fraction of H. pertussis in Mice

Dose mg.	Mouse No.	Body Weight			
		Initial g.	24 hours g.	48 hours g.	72 hours g.
1.5	1	26.0	23.0	22.6	22.3
	2	24.7	21.7	20.4	21.0
	3	23.0	21.5	20.3	20.8
	4	20.3	28.5	28.0	27.5
0.75	1	25.5	25.4	25.3	25.0
	2	25.8	22.9	22.2	23.7
	3	24.7	21.7	21.5	21.6
	4	21.0	19.3	18.3	18.0
0.375	1	28.1	25.2	25.5	25.7
	2	26.0	22.9	21.8	21.3
	3	25.3	23.5	22.8	22.3
	4	22.0	20.1	19.1	19.0
0.095	1	25.2	23.1	24.5	25.5
	2	29.3	27.0	27.7	28.7
	3	23.0	21.0	21.5	22.0
	4	23.0	20.0	20.8	21.9
0.048	1	23.2	21.6	20.9	22.7
	2	26.3	24.8	24.7	26.0
	3	22.3	20.5	20.3	21.8
	4	20.6	18.7	18.5	19.8
0	1	21.9	21.0	21.3	21.5
	2	24.4	24.6	24.6	24.5
	3	25.0	25.3	25.6	25.7
	4	26.2	26.1	26.3	26.8
	5	24.9	24.8	24.0	23.8
	6	24.0	24.4	24.2	25.7

The each dose was dissolve in buffered saline (phosphate buffer pH 8.3) and injected intraperitoneally in mice (0.2 ml.).

groups of five mice, weighing 22 ± 2 g. and minimum lethal dose (MLD) was determined as shown in Table II. The P_{105} fraction was most toxic, showing the 0.7 mg of MLD.

The amounts between 48 μ g. and 1.5 mg. of the Palc fraction were injected intraperitoneally into groups of four mice and the body weights were measured during the three days after injection. As seen in Table III, the marked decrease of body weight was observed even by injection of 48 μ g., although MLD of Palc was shown to be 6 mg.

Immunizing Potency—The amounts between 1 μ g. and 1 mg. of P_{105} were employed for immunization. The immunizing potency for mice was not observed, when immunity was measured by intraperitoneal challenge of 200 LD₅₀ of *H. pertussis* (strain 18323) ten days after immunization.

DISCUSSION

The two methods of Westphal, Lüderitz and Bister (9) and Perrault and Shear (10) had already been applied successfully to the preparation of lipopolysaccharides from a number of bacteria. These were unsuitable for the present study. The former gave a very viscous mixture when the equal volume of 90% phenol was not separated from phenol phase by centrifugation. By the latter, involved the treatment with low concentration of phenol (5 per cent) and 0.1 *M* trichloroacetic acid, any polysaccharide substances were not extracted.

A few improvements have been introduced in the method of preparation of lipopolysaccharide as follows. 1) Previous removal of protein from cells by extraction with 0.15 *M* saline. 2) The dispersion of the aqueous suspension of cells in Waring blender before the addition of phenol. 3) The addition of

lower concentration of phenol (one tenth volume of 90 per cent phenol). The polysaccharides were easily extracted by these improvements and purified by differential centrifugation, after ethanol precipitation.

The lipopolysaccharide (P_{105} fraction) thus obtained, was found to be toxic for mice but not to have immunizing potency.

The similar toxic lipopolysaccharide was obtained from the vaccine as well as living cells by this procedure. From these results, it is, in practice, desirable to remove or destroy this toxic lipopolysaccharide without loss of immunizing potency of vaccine. The study on the suitable conditions for this purpose is under investigation.

SUMMARY

1. The lipopolysaccharide was extracted from the *Hemophilus pertussis* by lower concentration of phenol.

2. The toxicity of this fraction for mice was confirmed by the lethality and the decrease of body weight after injection.

REFERENCES

- (1) Cruickshank, J. C., and Freeman, G. G., *Lancet*, **11**, 567 (1937)
- (2) MacLennan, A. P., *Biochem. J.*, **67**, 3 p (1957)
- (3) MacLennan, A. P., *Biochem. J.*, **74**, 398 (1960)
- (4) Eldering, G., *Am. J. Hyg.*, **36**, 294 (1942)
- (5) Eldering, G., Hornbeck, C., and Baker, J., *J. Bact.*, **74**, 133 (1957)
- (6) Akiya, S., Takahashi, H., Kuriyama, M., and Ogawa, N., *Japan. Med. J.*, **4**, 325 (1951)
- (7) Barton, C. J., *Anal. Chem.*, **20**, 1068 (1948)
- (8) Bryson, J. L., and Mitchell, T. J., *Nature*, **167**, 864 (1951)
- (9) Westphal, O., Lüderitz, O., and Bister, F., *Z. Naturforsch.*, **7B**, 148 (1952)
- (10) Perrault, A., and Shear, M. J., *Cancer Research*, **9**, 626 (1949)

Studies on the Amino Acids Present in Yeast RNA in Bound Form

II. Liberation of Amino Acids from RNA by Alkaline Treatment*

By SHUZO AKASHI and HANAKO ISHIHARA

(From the Department of Biochemistry, School of Medicine, Nagoya City University, Nagoya)

(Received for publication, February 27, 1961)

In a previous paper (1) it was reported that the ribonucleic acids which were prepared from various microorganisms and purified by the combined use of the Sevag method and the continuous paper electrophoresis technique proved to contain 7 to 9 amino acids, when hydrolyzed with 6 *N* HCl at 100°C for 20 hours. Their amino acid contents were determined to be approximately 1 per cent on the glycine basis. Suggestion has been made that these amino acids might exist as peptides in combination with the nucleic acid. In the succeeding study (2), various purified samples of yeast RNA from different sources were hydrolyzed with HCl under much milder conditions, liberating still 6 to 7 amino acids. This made it possible to consider that these amino acids might exist linked with RNA largely as single amino acids rather than as peptides.

The present work was carried out to see whether purified yeast RNA may separate amino acids by quite a mild alkali treatment. Evidence has been offered that the RNA can release amino acids which are to be divided into at least three groups by the ease of separability, indicating the possibility that there may be several at least three kinds of linkages between amino acids and RNA, differing in susceptibility to alkali. Data were also obtained in favor of the view that the amino acids may be in association with RNA in major part as peptides and in minor part as single amino acids.

EXPERIMENTAL AND RESULTS

Purified RNA employed for the present

study is the sample designated as sample No. 2 in the previous paper (2) *i.e.*, a commercial yeast RNA-Na (E. Merck) which was completely removed of protein by the Sevag method, followed by dialysis against distilled water at 2°C for three days.

The purified RNA preparation was successively treated with alkali of different concentrations which were raised in a triple manner such as, 0.01 *N*, 0.1 *N*, and 1 *N* KOH. The treatment with any of the alkali was carried out five times repeatedly, whereby digestion time was prolonged stepwise such as, *e.g.*, 0.5, 1.0, 2.0, 6.0 and 15 hours, the total period of time extending over about 24 hours. The details are as described below.

Estimation of Amino Acids and Inorganic Phosphate Liberated in the Dialyzates of Each Alkaline Digests—2.50 g. of the purified RNA were dissolved in 250 ml. of 0.01 *N* KOH and digested in a water-bath at 25°C for 30 minutes, after which the reaction mixture was placed in "Visking" dialysis tubing and dialyzed at 2~3°C against frequent change of distilled water until free of potassium ion. The combined dialyzates, in amount about 12 liters, were neutralized with perchloric acid and concentrated *in vacuo* to a volume of 25 ml. Insoluble potassium perchlorate separated by neutralization as well as by concentration was removed by filtration. One ml. of the concentrate was used for the determination of amino acids including peptides, according to the ninhydrin method of Moore and Stein with slight modification (3). Two ml. of the concentrate were analyzed for the content of inorganic phosphate by the method of Fiske and Subbarow. The solution remaining in the dialysis tubing

* This work was presented at the Biochemical Meeting of the 15th General Assembly of the Japan Medical Congress held at Tokyo in April, 1959.

was again made 0.01 *N* with respect to KOH and digested again at 25°C for 1 hour and dialyzed against distilled water until free of alkali. The combined dialyzates were analyzed for amino acid and phosphate. The tubing content, after four time repetition of 0.01 *N* KOH treatment, the digestion time being increased stepwise as mentioned above, was made 0.1 *N* with respect to KOH, and the digestion and dialysis procedures were carried out five times in a similar manner. The final tubing content remaining after 0.1 *N* KOH treatment was raised to an alkaline concentration of 1 *N* KOH and then subjected to the same digestion and dialysis procedures mentioned before, and the latter procedure was followed by assay of amino acids and phosphate with the dialyzate. The data obtained are summarized in Table I.

The above data are represented in the next diagram, in which the amount of amino acids and inorganic phosphate liberated by alkali of stepwise elevated alkalinity are plotted against the time of alkaline digestion.

Proof for the Presence of Peptides together with Amino Acids in the Dialyzates of Yeast RNA—This was established by the next two experi-

ments.

1) *Preliminary Test for Peptides*—The final dialyzates, namely, No. 5 dialyzate of each of the three fractions as designated I, II, and III in Table I was employed for this purpose. A given amount (1 ml.) of the dialyzates (25 ml.) was hydrolyzed with 3 *N* HCl at 100°C for 20 hours and upon removal of the HCl and ammonia by disillation *in vacuo*, the amino acids present were determined by the ninhydrin method. The amounts of amino acids in the dialyzate before and after acid hydrolysis were estimated, the data of which are recorded in Table II, indicating a considerable, *i.e.*, 2.5 to 6.2 fold increase in ninhydrin value after acid hydrolysis, therefore the presence of not a few peptides.

2) *Experiment by the Combined Use of Paper Chromatography and Paper Electrophoresis*—All of the remaining portion of No. 5 dialyzate of Fraction I (0.01 *N* KOH digest) was desalted by passing through resin columns of Dowex-I (OH) and Dowex-50(H). The final eluate was concentrated to an appropriate volume and applied to paper electrophoresis, yielding the result shown in Fig. 2-A.

The area of the above electrophoresis

TABLE I
Estimation of Amino Acids (Including Peptides) in Term of Glycine and Inorganic Phosphate Separated in the Dialyzates of Alkaline Digests of Yeast RNA (2.5 g.)

	Fraction I					Fraction II					Fraction III				
KOH concentration	0.01 <i>N</i>					0.1 <i>N</i>					1 <i>N</i>				
No. of dialyzate	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Time of digestion (hour)	0.5	1.0	2.0	6.0	18.0	0.5	1.0	2.0	6.0	15.0	0.5	1.0	2.0	6.0	15.0
Time from the start (hour)	0.5	1.5	3.5	9.5	27.5	0.5	1.5	3.5	9.5	24.5	0.5	1.5	3.5	9.5	24.5
Amino acid liberated* (μ g.)	431	548	356	330	258	287	523	454	237	232	736	1,299	1,178	701	707
" (mg./100g. RNA)	17.3	22.0	14.3	13.2	10.3	11.5	21.0	18.2	9.5	9.3	29.5	52.1	47.2	28.0	28.3
Total " (μ g.)	1,923					1,733					4,621				
" " (mg./100g. RNA)	77.1					69.5					185				
Inorganic P (μ g.)	6.38	1076	388	306	369	430	375	373	405	373	149	279	165	131	121
" (g./100g. RNA-P)	0.32	0.54	0.19	0.16	0.19	0.22	0.19	0.19	0.20	0.19	0.075	0.0141	0.083	0.066	0.061
Total inorg. P liberated (μ g.)	2,777					1,956					845				
" (g./100g. RNA-P)	1.40					0.99					0.43				

* Estimation of amino acids was carried out on the glycine basis, in which correction was made for the ninhydrin value due to the perchloric acid which was used for neutralization of alkali, since significant amounts of ninhydrin positive substance especially ammonia was present in the reagent.

pattern, which was corresponding to neutral amino acid and was assigned with an arrow mark, was cut off from the paper and extracted with water. The extract was developed by two-dimensional paper chromatography to find an unknown spot in addition to those spots for glycine, serine, valine, *etc.* as given in Fig. 2-B.

This unidentified spot suspected of peptide was cut off from the paper and extracted with water. The extract was hydrolyzed with 3 *N* HCl at 100°C for 20 hours. The

hydrolyzate was rechromatographed to identify the spot for serine, glycine, glutamic acid and histidine. The relevant chromatogram is illustrated in Fig. 2-C.

The same experiment was also made with each of No. 5 dialyzates of Fraction II (0.1 *N* KOH digest) and Fraction III (1 *N* KOH digest), obtaining results similar to those found with Fraction I.

Formation of Histidine Hydroxamate—One g. of purified RNA was dissolved in 4 ml. of water and 10 mg. of hydroxylamine hydro-

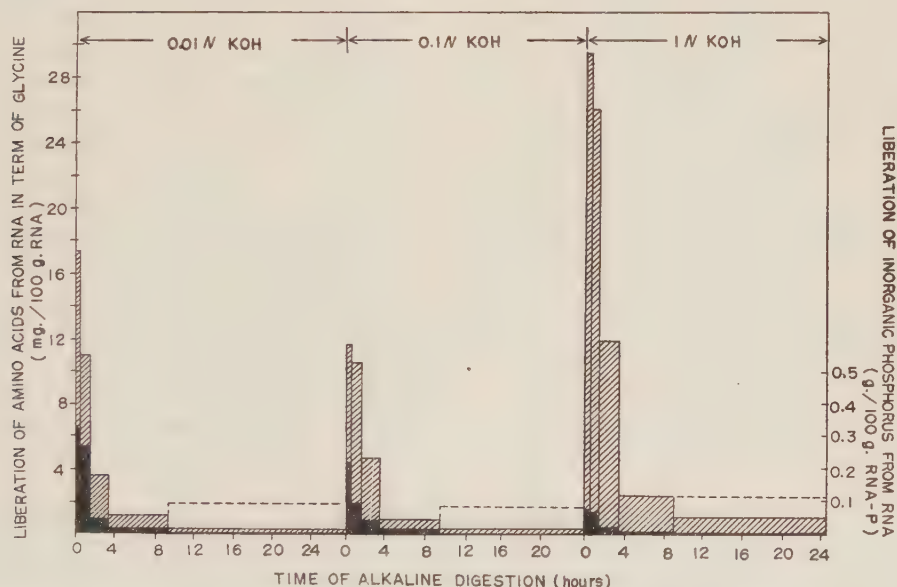


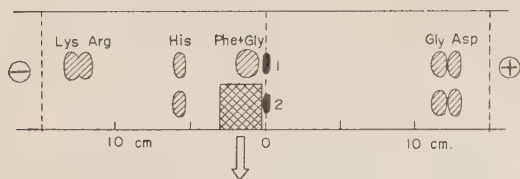
FIG. 1. Liberation of amino acids and inorganic phosphate from the purified yeast RNA by alkaline treatment, the alkalinity of which being elevated stepwise. The hatched areas denote amino acids liberated and the dark ones inorganic phosphate released. The area which is situated above No. 5 dialyzate of each fraction and enclosed by a dotted line indicates the increments of ninhydrin values produced by total acid hydrolysis of that portion of dialyzates.

TABLE II

Increase in Ninhydrin Values in Term of Glycine of Dialyzates by Acid Hydrolysis

	Dialyzate*		
	No. 5 (Fraction I)	No. 5 (Fraction II)	No. 5 (Fraction III)
	μg.	μg.	μg.
Ninhydrin value before acid hydrolysis	10.3	9.28	28.3
after acid hydrolysis	63.8 (10.3 × 1.2)	9.28 (9.28 × 5.4)	70.7 (28.3 × 2.5)

* Analysis was made using 1 ml. portion of each 25 ml. dialyzates.



Paper chromatography

FIG. 2-A. Paper electrophoresis pattern of No. 5 dialyzate of Fraction I (0.01 *N* KOH digest) of yeast RNA. Conditions: buffer pyridine-AcOH-H₂O (10:0.4:90) of pH 6.5, 800 volt, 45 min., 2 mA/cm., Toyo filter paper No. 50.

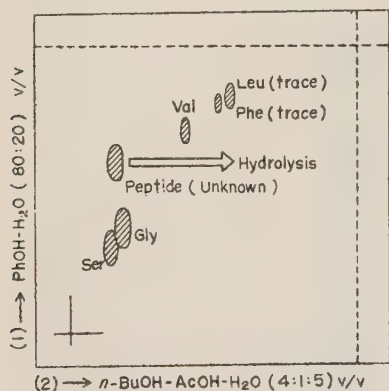


FIG. 2-B. Paper chromatogram of the portion corresponding to neutral amino acid separated by electrophoresis shown in Fig. 2-A.

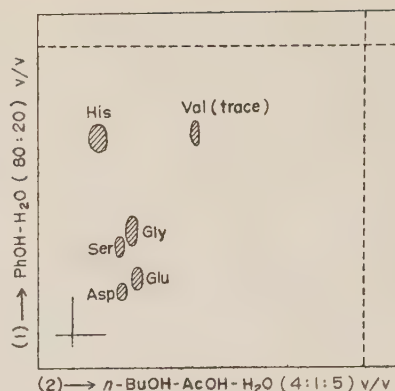


FIG. 2-C. Chromatogram of the Hydrolyzate of the extract from the peptide susceptible spot. Conditions: the same as in Fig. 2-B.

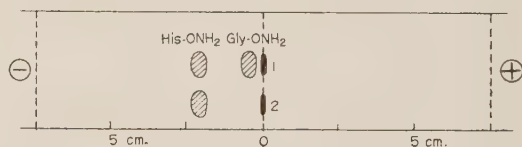


FIG. 3. Paper electrophoresis pattern of histidine hydroxamate (His-ONH₂). Condition: acetate buffer pH 5.5, 600 volt., 1.5 hours, 0.7 mA/cm., Toyo filter paper No. 50. Hydroxamates of glycine and histidine employed as check substance were prepared according to Safir and Williams (4).

chloride were added. The reaction mixture, upon adjustment of pH at about 5.5, was allowed to stand overnight at 2–3°C with occasional shaking. The solution was next dialyzed against distilled water and the dialyzate was evaporated *in vacuo* to dryness. The residue was dissolved in a little water and applied to paper electrophoresis. The pattern developed with ferric chloride solution revealed a single spot of reddish-brown color in accord with the check spot of histidine hydroxamate, as shown in Fig. 3.

DISCUSSION

It has been experimentally evidenced that the purified yeast RNA is able to separate very small amounts of amino acid as well as inorganic phosphate in three steps corresponding to varying alkalinity, when treated with dilute alkali such as, 0.01 *N*, 0.1 *N*, and 1 *N*

KOH at 20°C. The separated amount of amino acid in each of the three fractions I, II, and III was in the order of III>I>II, whereas that of inorganic phosphate was I>II>III.

The fact that amino acids appear forming three sharp peaks in the alkaline digest of RNA seems to suggest that at least three different alkali susceptible links may exist between amino acid and nucleic acid. With regards the inorganic phosphate, which was liable to be liberated mainly in Fraction I and II, is considered to be of alkali labile nature.

According to the work by Potter and Dounce (5), it was pointed out that firmly bound amino acids are present in the alkali stable fractions of the Schmidt-Thannhauser digest of RNA from calf pancreas, rabbit, and yeast. The amino acids were

postulated to be linked with phosphate group through their amino group as phosphoamide. However, they failed to observe amino acids in the mononucleotide, or alkali labile fraction. This alkali labile fraction was meant by those fractions which can be split off as mononucleotide, when RNA is treated with 1*N* NaOH at 37°C for 15 hours. In the present study, however, an alkali labile fraction does mean those fractions which are released by treatment with alkali having concentration equal to or smaller than 1*N* KOH, *e.g.*, 0.01*N* and 0.1*N* KOH at 20°C for about 24 hours.

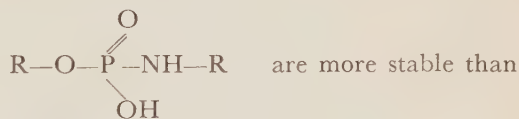
Concerning the other type of the bond involved between amino acids and the nucleotide, carboxy-phosphoryl or carboxy phosphoanhydride type was postulated as initial activation step of amino acid prior to protein synthesis by Hoagland (6). Meanwhile Lipmann *et al.* (7) made an experiment which demonstrated, using C¹⁴-labelled leucine, the formation of amino acid ribose ester bond in the soluble RNA of the rat liver, an important finding from the point of protein synthesis.

The other type of combination of amino acid with nucleic acid or nucleotide will be *via* pyrophosphate as found in uridine-diphosphate compounds. The latter compounds, however, usually readily release inorganic phosphate as well as component sugar by mild acid hydrolysis, but not by mild alkali treatment, since the glycosidic linkage between phosphoric acid and sugar is rather resistant to alkali. In this connection the work of this laboratory (8) should be noticed, according to which from a unique strain of *Alcaligenes foecalis* was isolated a peculiar kind of nucleic acid, conveniently named as "alcaligenic acid", and the substance contained about 8.5 per cent of sugar-peptide complex in bound form together with labile phosphate, both of which were split off when heated for 7 minutes at 100°C in 1*N* HCl. The present data which showed that mild alkali treatment of yeast RNA is able to liberate simultaneously both inorganic phosphate and amino acids may possibly be accounted for by assuming that the amino acids and pyrophosphate are bound

together either by an aminoacyl or an amido-phosphoryl group. Since no information is as yet available concerning such kind of compound, these types of combination seem to be possible, but less probable. Anyway, the nature and origin of alkali labile phosphate in yeast RNA still remain to be elucidated.

In view of those mentioned above, it appears likely that at least four types of linking may be involved between amino acid and RNA, namely, 1) N-phosphoryl type, 2) aminoacyl-phosphoanhydride type, 3) amino acid ribose ester type, and 4) diphosphate or pyrophosphate type. Moreover, the possibility cannot be excluded that amino acids are linked with the base components of RNA.

These various types seem to differ from each other in lability to alkali. Among them the most labile ones will be the aminoacyl-phosphoanhydride type and the diphosphate type because of their acid anhydride nature. The less labile one will be the N-phosphoryl type, since according to the study of Chantrenne (9) as cited by Potter and Dounce (5) the phosphoamide bonds of the type



the simple phosphoamide bonds that occur in phosphocreatine and phosphoarginine. The least labile one will be the ribose ester type. One of the reasons for this is, as reported by Lipmann (7), that between 2'/3'-leucine ester of AMP and leucy-AMP anhydride there is found considerable difference of reactivity of these amino acid derivatives with 1*M* hydroxylamine at pH 5.5 and 0°C, the former having values much lower than the latter.

Based on this premise the data of the present study can be accounted for as follows. Fraction III seems to include those amino acids which are not so readily liberated by dilute alkali, therefore being of the ribose ester type. Fraction I, on the contrary, may contain the most labile amino acids, accordingly, those belonging to the aminoacyl-phosphoanhydride and the diphosphate type. Fraction II, being the intermediate of Fraction

I and II, may represent the less labile amino acid involved in the N-phosphoryl type. Whether or not this conjecture is real, is a problem to be solved experimentally by future study.

However, it can be said undoubtedly that small amounts of amino acids bound to RNA are set free at least in three groups by various mild alkali treatments, owing to the different susceptibility to alkali of the linkages involved. A support for the presence of labile amino acid has been provided by the formation of histidine hydroxamate. However, none of the other amino acid hydroxamate has yet been obtained, for which further detailed studies are required. The reason for the hydroxamate formation limited to histidine may be partly due to the fact that histidine is apparently the predominant amino acid bound to the RNA in concern. That the free amino group is present in the various amino acids linked with RNA has been demonstrated by the unpublished data obtained in this laboratory, according to which dinitrophenylated RNA preparations were treated in a similar manner with different mild alkali, yielding several kinds of DNP-amino acids in the dialyzates.

With regard to the occurrence of peptide in bound form, the amino acid found in the RNA being studied seems to be associated in major part as peptide and in minor part as amino acid, as shown in Table II, which represents a considerable increase in ninhydrin value of No. 5 dialyzate of each fraction as a result of acid hydrolysis of the dialyzate. The study is now in progress, to elicit the nature of links between amino acid and nucleotide with the fraction which appears as nucleotides in Fraction III.

SUMMARY

1. Yeast RNA, completely purified by the Sevag method and prolonged dialysis, separated in the dialyzates very small amounts

of mixture of amino acid and peptide together with a trace of inorganic phosphate, forming three sharp peaks of Fraction I, II and III, when treated consecutively at 20°C with dilute alkali of varying concentrations such as, 0.01 *N*, 0.1 *N*, and 1 *N* KOH in a triple manner and each time dialyzed. Treatment with the same alkali concentration was carried out repeatedly five times, the digestion time of which being prolonged stepwise such as, 0.5, 1.0, 2.0, 6.0, and 15 hours.

2. The nature of links between amino acid and RNA involved in three fractions and also the origin of inorganic phosphate were conjectured.

3. Fraction I, the dialyzate of 0.01 *N* digest, comprising the most alkali labile amino acids, presumably deals with the aminoacyl-phosphoanhydride type and the diphosphate type. Fraction II, the dialyzate of 0.1 *N* KOH digest, being less alkali labile, may represent the N-phosphoryl type. Fraction III, the dialyzate of 1 *N* KOH digest, being relatively alkali stable, may include the amino acid ribose ester type.

4. A greater part of the bound amino acids seems to be in peptide form.

REFERENCES

- (1) Akashi S., and Yokoi H., *J. Biochem.*, **45**, 927 (1958)
- (2) Ishihara (Yokoi) H., *J. Biochem.*, **47**, 198 (1960)
- (3) Yokoi H., *J. Biochem.*, **46**, 133 (1959)
- (4) Safir S. R., and Williams J. H., *J. Org. Chem.*, **17**, 1298 (1952)
- (5) Potter J. L., and Dounce A. L., *J. Am. Chem. Soc.*, **78**, 3078 (1956)
- (6) Hoagland M. B., Stephenson M. L., Scott J. F., Hecht L. I., and Zamecnik P. C., *J. Biol. Chem.*, **231**, 241 (1958)
- (7) Zachau H. G., Acs G., and Lipmann F., *Proc. Natl. Acad. Sci.*, **44**, 88 (1958)
- (8) Akashi S., Yokoi H., and Goto H., *J. Biochem.*, **45**, 959 (1958)
- (9) Chantrenne H., *Publ. Staz. Zool. Napoli*, **23**, Suppl., P. 70

Adenosine Triphosphate and Shape of Erythrocytes

By MAKOTO NAKAO*, TOSHIKO NAKAO*, SABURO YAMAZOE
and HARUHISA YOSHIKAWA

(From the Department of Biochemistry, School of Medicine, Gunma University,
Maebashi, and Department of Nutrition and Physiological Chemistry,
Faculty of Medicine, The University of Tokyo, Tokyo)

(Received for publication, February 27, 1961)

The mechanism with which erythrocytes maintain their biconcave shape has remained to be clarified for a long time. An antispherizing factor which Furchgott and Ponder (1) once proposed was doubted by Ponder himself (2) later. Recently, Pranker (3) suggested that red blood cell envelopes might possess a certain contractile property, since the incorporation of P^{32} is low in the esters of the stroma obtained from those species with spheroidal red cells or from patients with hereditary spherocytosis.

In the course of the study on metabolism in preserved erythrocytes, following informations were obtained by the authors (4, 5): 1) Adenosine triphosphate (ATP) in erythrocytes, which had been almost entirely eliminated after long-term storage and could not be regenerated with the addition of inosine only, increased rapidly and occasionally rose above the normal level after incubation with the addition of both adenine and a purine nucleoside at 37°C. 2) Erythrocytes preserved for a long time are smooth spheres and, when they were incubated with adenine and nucleoside, they became crenated at first and changed to cap forms or became discoidal with the increase of ATP.

The present report is concerned with an experimental proof of the existence of an intimate relationship between the shape of erythrocytes and ATP level in them, using this technique of ATP regeneration in erythrocytes. A preliminary experiment along this line has

already been published elsewhere (6) and some of the present results were summarized briefly in the 8th Congress of International Congress of Haematology (7).

MATERIALS AND METHOD

Human red blood cells obtained by venipuncture were washed three times with physiological saline and suspensions of these cells in physiological saline with a definite amount of 0.154M NaF and in the same amount of physiological saline as a control were prepared.

Microscopical observation was carried out using hanging-drop-method.

After 10 ml. portions of about 30% cell suspension were incubated at 37°C under different conditions, each was centrifuged, the supernate discarded and 3% perchloric acid was added to the precipitate to the final volume of 12 ml. Five ml. of the supernate was neutralized with 5 ml of a dilute KOH solution, and $KClO_4$ precipitate was centrifuged off. Then, 5 ml. of the solution was analyzed by the Cohn and Carter's method using chromatography of Dowex 1 (Cl) (8).

RESULTS AND DISCUSSION

During incubation with a final concentration of $2 \times 10^{-2}M$ of sodium or potassium fluoride, the discoidal shape of the cells changed to a crenated disk at about two hours, a crenated sphere at about four hours, and after six hours almost all the cells were transformed to smooth spheres (Fig. 1A). On the other hand, when the cells were incubated with $5 \times 10^{-3}M$ of glucose and without addition of fluoride, their shape remained entirely unchanged even after six hours (Fig. 1B). When the cells were incubated without addition of either glucose or fluoride, the change of the shape to spheres was retarded (Fig. 1C).

* Present address: Department of Biochemistry, Faculty of Medicine, Yokohama University, Urahanecho, Minami, Yokohama, Japan.

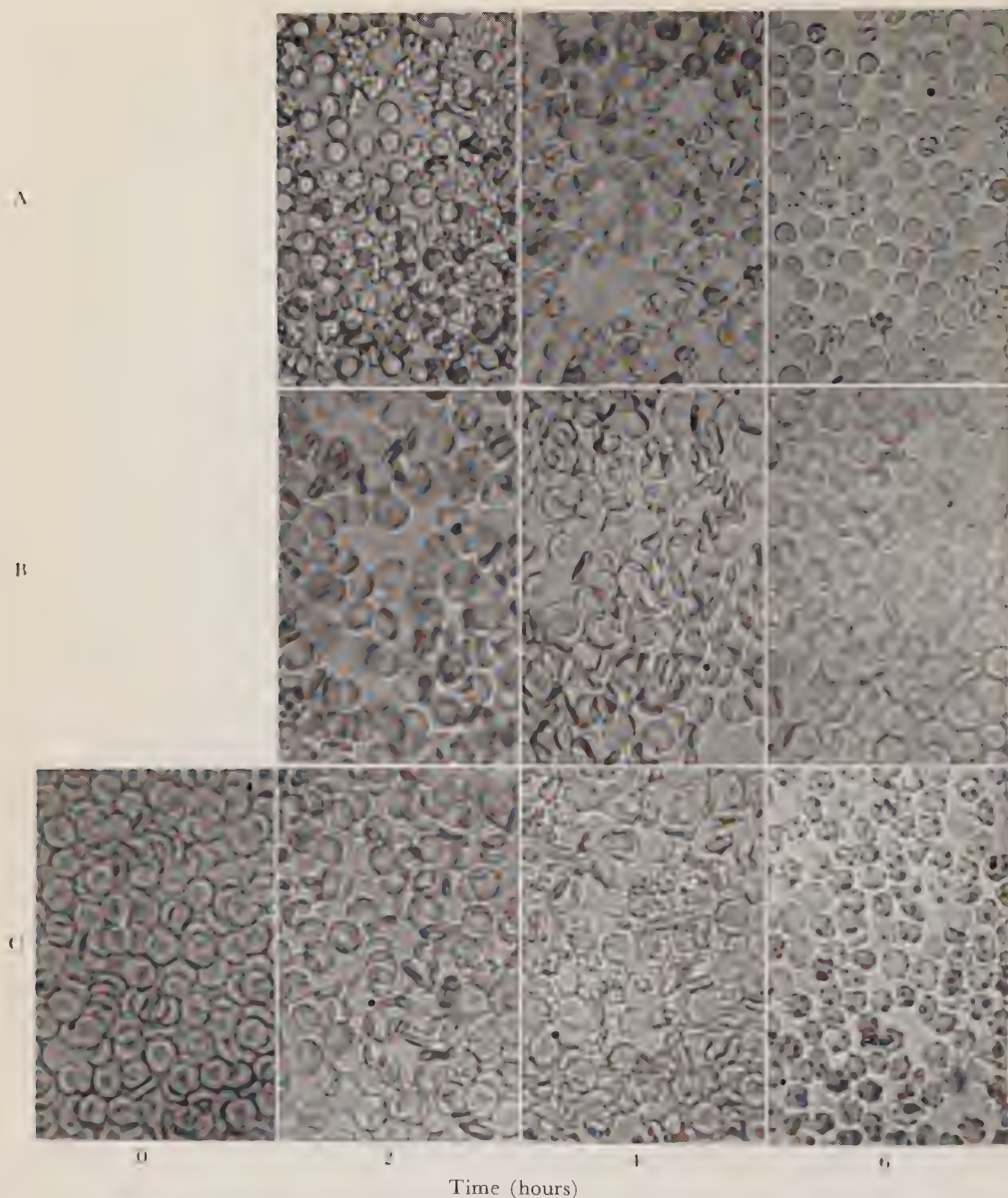


FIG. 1. Washed erythrocytes were incubated with NaF (A), glucose (B) and with saline alone (C), for 2, 4 and 6 hours.

Furthermore, smooth sphere cells obtained after fluoride-treatment for 6-7 hours were washed four times with cold saline in order to wash out fluoride thoroughly and incubated further in a medium with $5 \times 10^{-3}M$ glucose, $1 \times 10^{-2}M$ inosine and $2 \times 10^{-3}M$ adenine to restore the ATP content in the cells as in the previous experiments. Within one hour the

shape of the incubated cells turned to a crenated and then after 2 or 3 hours became a biconcave or a shallow cup form again (Fig. 2). The addition of inorganic phosphate and magnesium ion was occasionally effective in accelerating ATP synthesis and transformation of the shape, on account of the compensation of the loss of these ions which flowed out

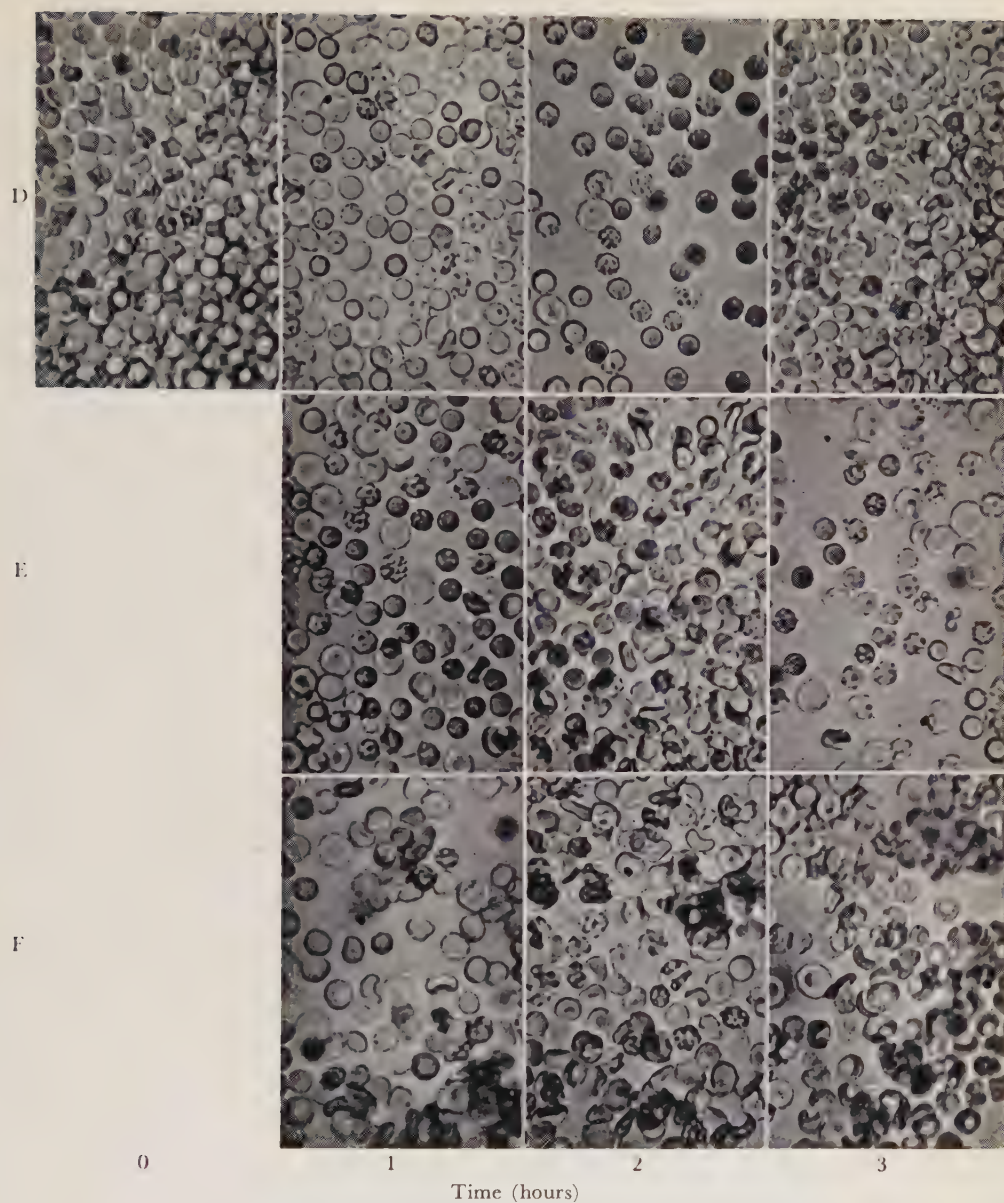


FIG. 2. Erythrocytes treated with NaF for 6 hours were washed to eliminate NaF, and reincubated with saline alone (D), with glucose (E) and with glucose, inosine and adenine (F).

from inside the cells during preincubation and washing (Fig. 3). The disk-sphere transformation and vice versa could be repeated several times. The phenomenon was also observed in red cells obtained from preserved human blood or from rat or pig blood as well.

In parallel with the morphological observation described above, the content of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) in the cells were estimated according to Cohn and Carter (8).

The data are shown in Fig. 4 and 5. The abscissa indicates the concentration of

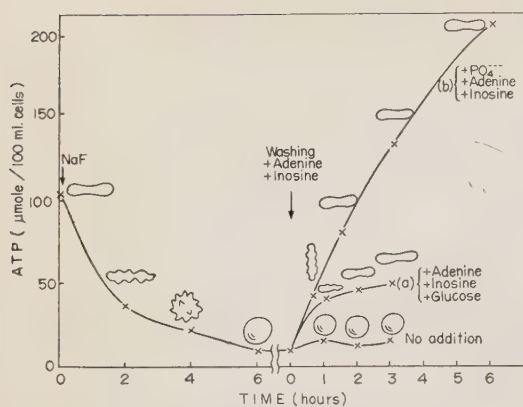


FIG. 3. Washed erythrocytes were incubated with NaF at 37°C for 6 hours and after washing out fluoride, reincubation was carried out with addition of inosine, adenine and glucose (a), and with addition of adenine, inosine and inorganic phosphate (b).

ATP or ADP and the ordinate, the percentage of disk, crenated and smooth spheres respectively throughout the whole population of the cells. As seen in Fig. 4, when ATP level was above a half of normal content, or about 50 μ mole per 100 ml. of cells, erythrocytes were discoidal as control erythrocytes; when ATP level was below one tenth of the original level, about 15 μ mole per 100 ml. of cells, they became smooth spheres; and when the amount of ATP was between these two levels, they were crenated. However, as Fig. 5 indicates there was no correlation between ADP level and the shape of erythrocytes.

Although the amount of total adenine nucleotide was emphasized in the preliminary report (6), the amount of ATP exactly estimated seems to have a more direct relation-

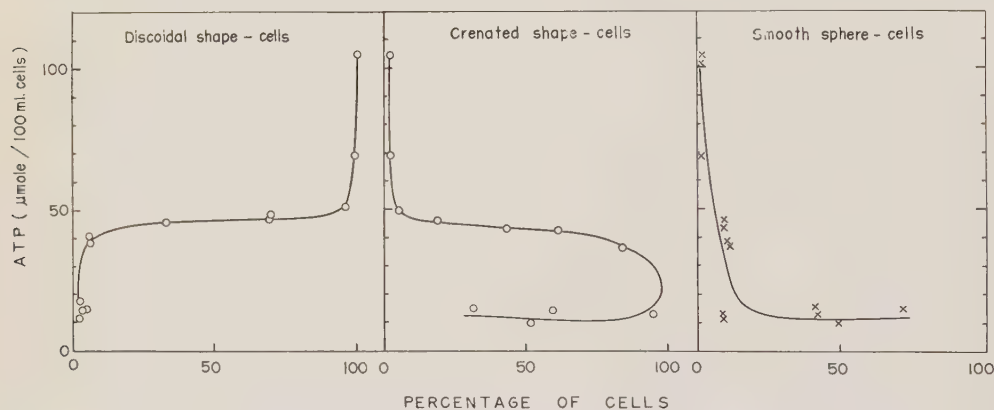


FIG. 4. Adenosine triphosphate level in erythrocytes and their shape. Abscissa: Percentage of the cells with various shapes. Ordinate: The content of ATP in erythrocytes.

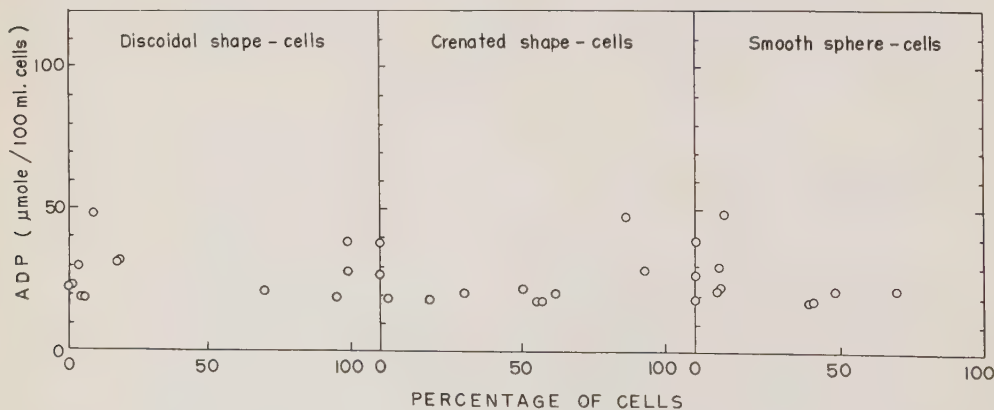


FIG. 5. Adenosine diphosphate level in erythrocytes and their shape. Abscissa: Percentage of the cells with various shapes. Ordinate: The content of ADP in erythrocytes.

ship to the shape.

A similar transformation was also observed during the preservation of blood in Acid-Citrate-Dextrose-Medium at 4°C: the biconcave disks changed to crenated disks at first after several days and then to crenated spheres and finally to smooth spheres after more than several weeks. Calculated from the results which were obtained from gradient elution technique of ion exchange column chromatography on Dowex 1, adenosine triphosphate contents of the cells and photographs of the preserved erythrocytes are shown in Fig. 6. Each cell shape type was quite the same whether it was observed at 2°, 20° or 37°C.

The observations presented here lead us to the following conclusions:

1. If ATP content in red cells is over a certain level, that is about 50 per cent of the original level, the normal shape of the cells is maintained, and if it is below this level the shape becomes crenated, and when it decreases to below about 10 per cent, the shape of the cells changes to a perfect sphere.

2. The presence of plasma protein is not necessary for the red cells to maintain their discoidal shape. Therefore, the presence of antisphering factor in plasma was decisively denied.

3. Other workers have reported that change of pH and ionic strength causes a similar transformation (10, 11). In this experiment, however, ATP level gives rise to the disk-sphere and also spherical shape-disk

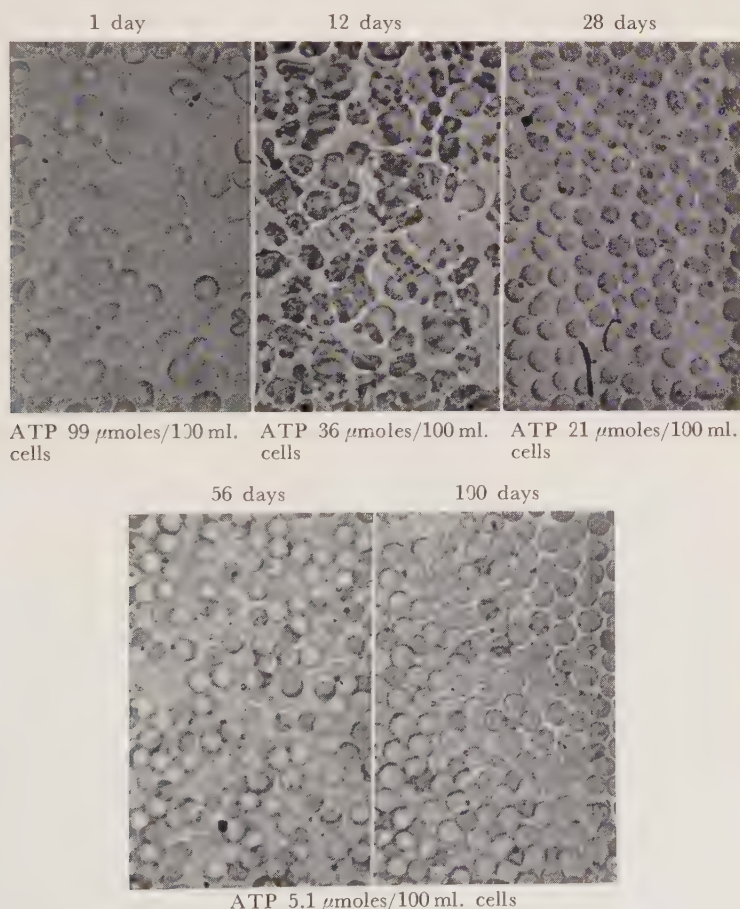


Fig. 6. Erythrocytes preserved in Acid-Citrate-Dextrose-Medium for 12, 28, 56 and 100 days.

transformation of red cells, while pH, cation concentration and ionic strength remained unchanged.

4. The maintenance of the shape of the red cells may depend not on the turnover of ATP, but on the level of ATP, because the cell shape remained quite unaffected by a temperature change alone.

The fact that ghost obtained from long-stored red cells changed to a goblet-like shape with the addition of ATP in the final concentration of 0.03–0.04 *M* or higher, differing in this respect from intact erythrocytes, has been previously reported by the authors (12).

The observations provide experimental evidence for the assumption that the shape of the erythrocytes is dependent upon ATP level in the cells under normal conditions. The observed phenomena might also recall the relation between the plasticity of muscle and its relatively high ATP content. It might be a general rule that ATP is necessary for the manifestation of the normal shape in various types of the cells.

The extraction of ghost protein is now in progress in our laboratory and will be reported

elsewhere.

REFERENCES

- (1) Furchgott, R. F., and Ponder, E., *J. Exptl. Biol.*, **17**, 117 (1940)
- (2) Ponder, E., *Blood.*, **9**, 227 (1954)
- (3) Pranker, T. A. J., *Biochem. J.*, **58**, 622 (1954)
- (4) Nakao, M., Nakao, T., Tatibana, M., Yoshikawa, H., and Abe, T., *Biochim. et Biophys. Acta*, **32**, 564 (1959)
- (5) Nakao, M., Nakao, T., Tatibana, M., and Yoshikawa, H., *J. Biochem.*, **47**, 661 (1960)
- (6) Nakao, M., Nako, T., and Yamazoe, S., *Nature*, **187**, 945 (1960)
- (7) Nakao, M., Nakao, T., Yamazoe, S., and Yoshikawa, H., 'Proceedings of the VIIIth Congress of International Haematology' (1960) (in press).
- (8) Cohn, W. E., and Carter, C. E., *J. Am. Chem. Soc.*, **72**, 4273 (1950)
- (9) Kuroda, K., and Mishiro, Y., *Tokushima J. Exptl. Med.*, **2**, 1, (1955)
- (10) Ponder, E., "Hemolysis and Related Phenomena," Grune & Stratton, New York (1948)
- (11) Kuroda, K., and Mishiro, Y., *Tokushima J. Exptl. Med.*, **1**, 153 (1954)
- (12) Nakao, M., Nakao, T., Tatibana, M., and Yoshikawa, H., *J. Biochem.*, **47**, 694 (1960)

The Use of Oligomycin as an Inhibitor of Oxidative Phosphorylation

By F. HUIJING and E. C. SLATER

(From the Laboratory of Physiological Chemistry, University of Amsterdam,
Amsterdam, Netherlands)

(Received for publication, April 4, 1961)

Over the past 50 years the study of the mechanism of intracellular respiration has been greatly facilitated by the introduction of respiratory inhibitors which react selectively at one point in the chain of enzymes and carriers which are responsible for the oxidation reactions. The most useful inhibitors used at present are cyanide and carbon monoxide (1) which combine with cytochrome a_3 (2) at the oxygen end of the chain, antimycin (3, 4) which reacts in the middle of the chain and Amytal (5) which reacts nearer the substrate end of the chain in the region of the DPNH-oxidizing flavoprotein. Amytal is useful since in concentrations of about 1 mM it almost completely inhibits the oxidation of DPN⁺-requiring substrates without having any effect on the oxidation of those substrates like succinate and glycerol phosphate which enter the respiratory chain by direct reaction with flavoprotein.

All four of the inhibitors mentioned above are effective with both phosphorylating and non-phosphorylating preparations of the respiratory chain. Höllunger (6) introduced a new type of selective inhibitor, guanidine, which inhibited respiration coupled with phosphorylation, without having any effect on the non-phosphorylating respiration of mitochondrial fragments or of mitochondria in the presence of dinitrophenol. Guanidine has not been widely used, probably because the mitochondria must be preincubated with rather high concentration for complete inhibition. More recently, Lardy, Johnson and McMurray (7) have introduced the antibiotic oligomycin which, in very low concentrations, has effects similar to those brought about by guanidine.

The starting point of the present investigation was the report by Lardy *et al.* (7) that oligomycin inhibited the oxidation of succinate by rat-liver mitochondria by only 60 per cent, although the oxidation of glutamate was completely inhibited, while the phosphorylation associated with the residual respiration of succinate was completely inhibited. As the investigation proceeded the great value of oligomycin as a tool in the study of coupled oxidations became apparent. It is the main purpose of the present paper to draw attention to this fact.

METHODS

Rat-liver mitochondria were isolated by the method of Hogeboom (8) as described by Myers and Slater (9), rat-heart sarcosomes by the method of Cleland and Slater (10). The heart-muscle preparation (11) was prepared from horse heart as described by Slater (12). Hexokinase was isolated by the method of Darrow and Colowick (13); its activity is expressed in the units of Berger *et al.* (14).

Oxygen uptake, except with ascorbate as substrate, was measured at 25°C with differential manometers, with a constant of about 0.7 μ l. O₂/mm. manometer fluid. The reaction volume was 1 ml. The centre well contained 0.1 ml. 2M KOH and a roll of filter paper. When oxidative phosphorylation was measured the reaction was stopped with trichloroacetic acid and the esterified phosphate measured by Method II of Slater (15).

The ATPase activity was measured at room temperature (19±1°C) as described by Myers and Slater (9), except that Tris-HCl was used as buffer.

The ADP-ATP exchange reaction was measured essentially as described by Wadkins and Lehninger (16). 8-C¹⁴-ADP was prepared from 8-C¹⁴-ATP (Schwarz) by reaction with hexokinase and glucose. After adding trichloroacetic acid and serum albumin, carrier ADP was added to the protein-free supernatant

and the ADP was purified by adsorption on washed charcoal and elution with 50% ethanol-0.5% NH₄OH (sp. gr. 0.880). The preparation was free from ATP or AMP detectable by paper electrophoresis. In the protein-free extracts obtained after an experiment in which the ADP \rightleftharpoons ATP exchange was studied, ADP was separated from ATP by paper electrophoresis in a water-cooled electrophoresis apparatus (E. C. Apparatus Co.), containing 6.4 \times 50 cm. strips of Schleicher & Schüll SS 598 paper in 0.08 M citrate buffer, pH 5.8. Running for 3 hours at 9 volts/cm. was sufficient to separate ATP, ADP and AMP. The nucleotides were eluted with 3 ml. water and a sample counted after evaporation on stainless-steel pans in a Nuclear Chicago D 47 gas-flow counter with a very thin mica window.

Protein was determined by the biuret method as described by Cleland and Slater (10).

Antimycin was kindly supplied by the Kyowa Fermentation Company, oligomycin was kindly supplied by Dr. J. Links. A molecular weight of 333 was assumed for the oligomycin (Lardy *et al.* (7) state that 2 μ g./ml. = 6×10^{-6} M).*

RESULTS

Effect of Oligomycin on Oxidation of Glutamate, Succinate and Ascorbate—In agreement with Lardy *et al.* (7), oligomycin was found to inhibit the oxidation of glutamate by more than 90 per cent, while the inhibition of succinate oxidation was incomplete, amounting to 75 per cent in the experiment shown in Table I. A concentration of 0.75 μ g./ml. oligomycin (1.4 μ moles/g. protein) was

sufficient for maximum inhibition. Since a large part of the O₂ uptake measured when liver mitochondria are oxidizing relatively low concentrations of succinate is due to oxidation past fumarate (17), it was important to know whether the oxidation step succinate \rightarrow fumarate was at all inhibited by oligomycin. This was studied by using 2.1 mM Amytal to inhibit the further oxidation of the fumarate. The experiment summarized in Table II shows that, under these conditions also, oligomycin inhibited succinate oxidation and that the inhibition was incomplete, varying between 54 per cent. and 76 per cent in different experiments.

The results shown in Table II (for succinate) and Table III (glutamate) confirm the important finding of Lardy *et al.* (7) that the inhibition of respiration can be prevented by dinitrophenol. In contrast dinitrophenol has no effect on the inhibition by antimycin. In the presence of dinitrophenol, even 4 μ g./ml. oligomycin had no effect on the oxidation of succinate. Fig. 1 shows that the inhibition of succinate oxidation by oligomycin (54 per cent in this experiment) disappears completely when dinitrophenol is added to the reaction mixture. Table IV shows that low concentrations of dinitrophenol are sufficient.

Effect of Oligomycin on Oxidative Phosphorylation—In agreement with Lardy *et al.* (7) it was found that 0.75 μ g./ml. oligomycin

TABLE I

Inhibition by Oligomycin of Oxidation of Glutamate and Succinate by Rat-liver Mitochondria

15 mM KCl, 2 mM EDTA, 20 mM glucose, 25 mM Tris-acetate buffer (pH 7.4), 30 mM phosphate, 5 mM MgCl₂, 0.1 mM ATP, 4% ethanol (from the oligomycin), 50 mM sucrose, 0.01 M succinate or 0.02 M L-glutamate, 150 units hexokinase, 1.6 mg. mitochondrial protein.

Oligomycin		Glutamate		Succinate	
(μ g./ml.)	μ moles/mg. mitochondrial protein	μ l. O ₂ /h.	Inhibition (%)	μ l. O ₂ /h.	Inhibition (%)
0	0	116	—	230	—
0.25	0.5	104	10	212	8
0.75	1.4	11	91	58	75
2.25	4.2	9	92	56	76

* Note added in proof (April 22, 1961). The properties of oligomycin are reviewed by J. Visser, D. E. Weinauer, R. C. Davis and W. H. Peterson (*J. Biochem. Microbiol. Techn. Eng.* 2, 31 (1960)) which has just come to our notice. It consists of a complex of three structurally related components (A, B and C) of molecular weights 424, 394 and 478, respectively.

TABLE II

Inhibition by Oligomycin and Antimycin of Oxidation of Succinate by Rat-liver Mitochondria, in the Presence of Amytal, and in Presence and Absence of Dinitrophenol

The conditions were the same as in Table I, except that ethanol was 5% and 2.1 mM Amytal was also present; 1.75 mg. mitochondrial protein.

Oligomycin		Antimycin (μ mole/g. protein)	without dinitrophenol		with 0.1 mM dinitrophenol	
(μ g./ml.)	(μ mole/g. protein)		μ l. O ₂ /h.	Inhibition (%)	μ l. O ₂ /h.	Inhibition (%)
0	0	0	149	—	140	—
0.75	1.3	0	64	57	129	8
1.50	2.6	0	60	60	158	—13
0	0	0.6	8	95	81	94

TABLE III

Inhibition by Oligomycin and Antimycin of Oxidation of Glutamate by Rat-liver Mitochondria, in Presence and Absence of Dinitrophenol

The conditions were the same as Table I except ethanol, 3% and Tris-HCl buffer instead of Tris-acetate; mitochondrial protein, 1.55 mg.

Oligomycin		Antimycin (μ mole/g. protein)	without dinitrophenol		with 0.1 mM dinitrophenol	
(μ g./ml.)	(μ mole/g. protein)		μ l. O ₂ /h.	Inhibition (%)	μ l. O ₂ /h.	Inhibition (%)
0	0	0	163	—	198	—
0.75	1.4	0	12	93	202	—1
1.50	2.9	0	19	88	212	—7
0	0	0.7	12	93	10	95

TABLE IV

Effect of Dinitrophenol Concentration on Oxidation of Succinate (in presence of Amytal) by Rat-liver Mitochondria in Presence and Absence of Oligomycin

The conditions were the same as in Table II, except ethanol, 2%; Amytal, 0.7 mM; mitochondrial protein, 1.7 mg.

Dinitrophenol (μ M)	without oligomycin		with oligomycin (1 μ g./ml.)	
	μ l. O ₂ /h.	P : O	μ l. O ₂ /h.	% Inhibition
0	245	1.66	60	75
1	239	0.99	80	67
5	250	0.74	104	58
10	243	0.50	242	0

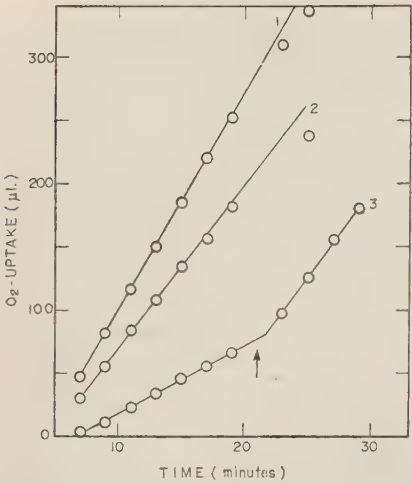


FIG. 1. Relief by dinitrophenol of inhibition by oligomycin of oxidation of succinate. The conditions were the same as in Table II, except pH 7.0; ethanol, 2%; mitochondrial protein, 1.3 mg. Curve 1, no further addition; Curve 2, 0.7 mM Amytal; Curve 3, 0.7 mM Amytal and 1 μg./ml. oligomycin; 0.2 mM dinitrophenol added at arrow.

completely inhibited the phosphorylation associated with the oxidation of succinate by rat-liver mitochondria and rat-heart sarcosomes.

TABLE V

Effect of Oligomycin on Phosphorylation Coupled with the Oxidation of Ascorbate (in presence of cytochrome c) by Rat-heart Sarcosomes

Rat-heart sarcosomes were treated hypotonically by Procedure (b) of Slater (18). 30 mM potassium phosphate, 0.2 mM ADP, 0.2 mM AMP, 5 mM MgCl₂, 1 mM EDTA, 20 mM glucose, 10 mM potassium ascorbate, 28 μM cytochrome c, 100 units hexokinase, 0.6–2.8% ethanol. 0.2 mg. sarcosomal protein/ml. Reaction vol., 3.15 ml. 20 min. at 25°C.

Oligomycin (μg./ml.) (μmoles/g. protein)		Δ O μatoms	Δ est. P μmoles	P : O
0	0	3.68	1.30	0.35
0.64	10	3.81	0	0
1.91	29	3.77	−0.10	0
2.85	43	3.62	−0.15	0

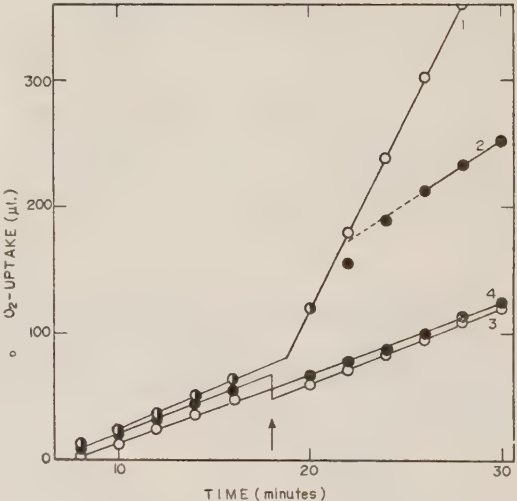
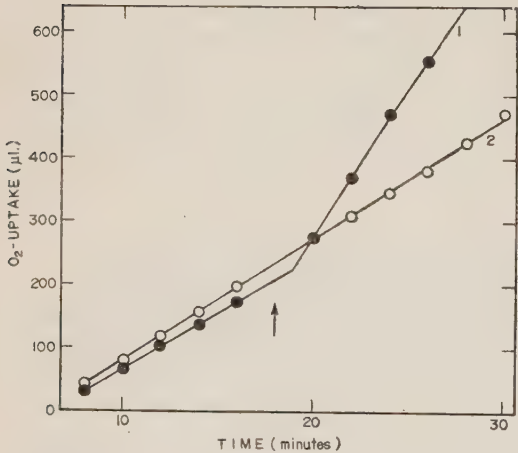


FIG. 2. Effect of oligomycin on rate of oxidation of succinate or glutamate by rat-liver mitochondria in the absence of ADP.

A. The conditions were the same as in Table II except ethanol, 3%; mitochondrial protein, 2.7 mg.; no hexokinase before tip. At arrow, 150 units hexokinase added. Curve 1, no oligomycin; Curve 2, oligomycin, 0.75 μg./ml.

B. The conditions were the same as in A (same mitochondrial preparation), except glutamate (20 mM) in place of succinate. Curve 1, no oligomycin, hexokinase (150 units) added at arrow; Curve 2, no oligomycin, 2 μmoles ADP added at arrow; Curve 3, 0.75 μg./ml. oligomycin; hexokinase added at arrow; Curve 4, 0.75 μg./ml. oligomycin; ADP added at arrow. (The breaks in Curves 3 and 4 at the time of addition are due to a "tipping artefact")

It was also found (Table V) that phosphorylation coupled with the oxidation of ascorbate (in the presence of cytochrome *c*) by swollen rat-heart sarcosomes (18) was completely inhibited by oligomycin. The O_2 uptake was not affected.

Effect of Oligomycin on Respiration in the Absence of ADP or Inorganic Phosphate—Lardy and Wellman (19) showed that both ADP and inorganic phosphate are required for maximum respiration by rat-liver mitochondria. Although the degree of stimulation of the respiration by adding ADP to an ADP-deficient system or by adding phosphate to a phosphate-deficient system is always several-fold, there is nevertheless an appreciable "basal" respiration especially with succinate as substrate (see, for example, Slater and Hülsman (20)).

Fig. 2 shows that oligomycin has no effect on this basal respiration measured in the absence of ADP, with either glutamate or succinate as substrate for rat-liver mitochondria. Oligomycin was also found to have no effect on the rate of oxidation of glutamate or succinate measured in the absence of inorganic phosphate with either rat-liver or rat-heart sarcosomes. Fig. 3

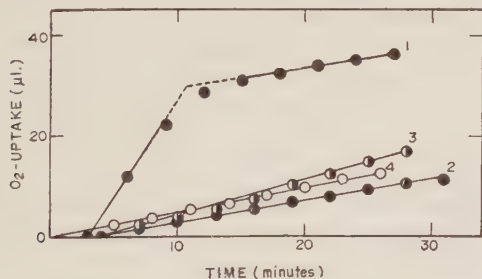


FIG. 3. Effect of oligomycin on rate of oxidation of glutamate by rat-heart sarcosomes in the absence of inorganic phosphate. 15 mM KCl, 2 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.9 mM ADP, 20 mM glucose, 5 mM $MgCl_2$, 20 mM L-glutamate, 1% ethanol, 100 units hexokinase, 1.2 mg. sarcosomal protein in all flasks. Curve 1, 10 mM phosphate also present (the sharp break in the curve corresponds to the exhaustion of the phosphate); Curve 2, no further addition; Curve 3, 1 $\mu g./ml.$ oligomycin, 10 mM phosphate present; Curve 4, 1 $\mu g./ml.$ oligomycin, no phosphate.

illustrates this for rat-heart sarcosomes oxidizing glutamate.

The rate of oxidation of succinate by the non-phosphorylating Keilin and Hartree heart-muscle preparation was not inhibited by up to 30 $\mu g./ml.$ oligomycin; the DPNH oxidase of this preparation was inhibited about 10 per cent (Colpa-Boonstra, unpublished).

In the absence of phosphate, the oxidation of substrates by rat-liver mitochondria can be stimulated by arsenate (21-23). This arsenate-stimulated oxidation was also found to be inhibited by oligomycin. In contrast to dinitrophenol, arsenate was unable to release the inhibition by oligomycin either in the absence or presence of phosphate (see Fig. 4).*

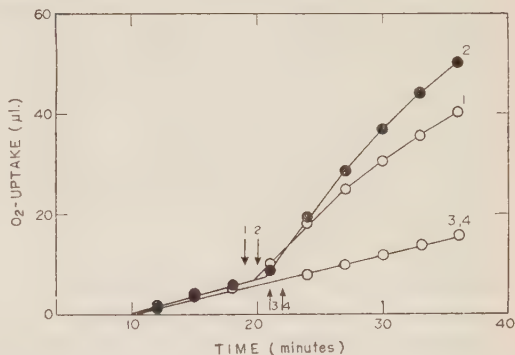


FIG. 4. Effect of arsenate in the presence and absence of phosphate on the inhibition by oligomycin of the oxidation of glutamate by rat-liver mitochondria. The conditions were the same as in Fig. 3. 4.1 mg. mitochondrial protein. Curve 1, 30 mM arsenate tipped at Arrow 1; Curve 2, 30 mM arsenate + 10 mM phosphate tipped at Arrow 2; Curve 3, 1 $\mu g./ml.$ oligomycin present and 30 mM arsenate tipped at Arrow 3; Curve 4, 1 $\mu g./ml.$ oligomycin present and 30 mM arsenate + 10 mM phosphate added at Arrow 4. Curves 3 and 4 are practically identical. Before the tip, all four curves were practically identical.

From the experiments described to date it is clear that oligomycin inhibits that fraction of the respiration which is coupled with

* Estabrook (34) has very recently reported similar results obtained with the use of the oxygen electrode.

phosphorylation, but the non-coupled respiration is not inhibited. This is the case whether uncoupling is brought about by addition of dinitrophenol or by fragmentation of the mitochondria (*e.g.* the Keilin and Hartree heart-muscle preparation). The non-coupled respiration of mitochondrial preparations is probably due to partially fragmented mitochondria which they contain. Uncoupling by the addition of arsenate, however, does not relieve the inhibition by oligomycin.

Effect of Oligomycin on the ATPase of Mitochondrial Preparations—Freshly prepared rat-liver mitochondria have no appreciable ATPase activity at pH 7.0 but do show some activity at higher pH's with a pH optimum at 9.4 (Myers and Slater (9)). In agreement with Lardy *et al.* (7) it was found that oligomycin, unlike dinitrophenol, did not induce any ATPase activity at pH 7.0. Further, it was found that the activity at pH 9.0 was completely inhibited by 0.75 $\mu\text{g./ml.}$ oligomycin.

Also in agreement with Lardy *et al.* (7) the dinitrophenol-induced ATPase activity of

TABLE VI

Effect of Oligomycin on Dinitrophenol-induced ATPase Activity of Rat-liver Mitochondria

0.05 *M* Tris-HCl buffer, 0.075 *M* KCl, 2 *mM* ATP, 0.5 *mM* EDTA, 2.7% ethanol (from the oligomycin solution), 0.2 *mM* 2,4-dinitrophenol at pH 7.0, 2.0 *mM* at pH 9.0; 3.5 mg. mitochondrial protein. Reaction vol., 1.5 ml.; reaction time, 15 min.

Oligomycin ($\mu\text{g./ml.}$) ($\mu\text{moles/g.}$ protein)		ATPase activity (μmoles P/h./mg. protein) at	
		pH 7.0	pH 9.0
0	0	8.7	12.0
0.25	0.32	8.5	10.5
0.50	0.64	6.0	8.3
0.75	0.96	6.4	7.8
1.00	1.29	5.3	6.7
1.50	1.93	3.5	5.7
2.00	2.57	1.4	4.3

rat-liver mitochondria was inhibited by oligomycin but rather higher concentrations were necessary than in the absence of dinitrophenol (see Table VI). The effect of

TABLE VII

Effect of Oligomycin on Mg^{2+} -stimulated ATPase Activity of Keilin and Hartree Heart-muscle Preparation

2 *mM* ATP, 50 *mM* Tris-HCl buffer, 75 *mM* KCl, 0.5 *mM* EDTA, 70 *mM* sucrose, 1.6 *mM* MgCl_2 , 0.6 mg. protein.

Oligomycin ($\mu\text{g./ml.}$) ($\mu\text{moles/g.}$ protein)		ATPase activity (μmoles P/h./mg. protein) at	
		pH 7.0	pH 9.0
0	0	11.5	16.6
0.33	2.5	3.2	5.1
0.66	5.0	1.3	1.9
1.00	7.5	1.9	0.6
1.30	9.7	0	0.6
2.00	15	0	—

TABLE VIII

Effect of Oligomycin on $\text{ATP} \rightleftharpoons \text{ADP}$ Exchange Reaction Catalysed by Rat-liver Mitochondria

5 *mM* ATP (Sigma), 1 *mM* 8- C^{14} -ADP, 75 *mM* KCl, 0.5 *mM* EDTA, 1.5 *mM* MgCl_2 , 37.5 *mM* Tris-HCl buffer (pH 7.0) in a final volume of 0.5 ml. The reaction was begun by adding 0.5 mg. mitochondrial protein. After 20–25 min. at room temperature, 0.1 ml. 20% HClO_4 was added. ADP and ATP were separated in the neutralized supernatant by paper electrophoresis, and counted as described under METHODS.

	c.p.m. in ATP	c.p.m. in ADP	c.p.m. in $\text{ATP} \times 100$ c.p.m. in $(\text{ADP} + \text{ATP})$
Expt. 1			
Zero-time control	25	304	8
20-min. reaction without oligomycin	264	68	79
20-min. reaction with 0.66 $\mu\text{g./ml.}$ oligomycin	221	60	79
Expt. 2			
25-min. reaction without oligomycin	319	69	82
25-min. reaction with 0.66 $\mu\text{g./ml.}$ oligomycin	312	64	83

oligomycin was approximately the same at

both pH's. [It should be noted that the concentrations of dinitrophenol chosen were those giving optimal ATPase activities at the two pH's, *viz.* 0.2 mM at pH 7.0 and 2 mM at pH 9.0 (Hemker and Hülsmann (24))].

The Mg^{2+} -stimulated ATPase of the Keilin and Hartree heart-muscle preparation was completely inhibited, both at pH 7 and 9, by low concentrations of oligomycin (Table VII).

Effect of Oligomycin on $ADP \rightleftharpoons ADP$ Exchange Reaction—In agreement with Lardy (35; and personal communication) it was found that oligomycin had no effect on the $ADP \rightleftharpoons ATP$ exchange reaction (Table VIII).

DISCUSSION

It is interesting to compare the actions of antimycin, oligomycin and dinitrophenol on mitochondrial respiration.

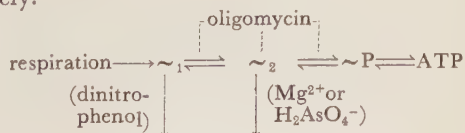
Antimycin inhibits the respiration of mitochondrial fragments such as the Keilin and Hartree heart-muscle preparation virtually completely and that of phosphorylating mitochondria almost completely. Its effect is localized between cytochromes *b* and *c*₁ in the respiratory chain (4, 25). It has no effect on the oxidative phosphorylation associated with that portion of the chain which it does not inhibit *e.g.* between cytochrome *c* and oxygen (26, 27, 18). The inhibition is not relieved by dinitrophenol. Antimycin can be considered simply as a respiratory inhibitor, which blocks the main respiratory chain. Inhibition by antimycin can be by-passed by a slow route in mitochondrial preparations, so that inhibition is never complete (28).

Dinitrophenol does not inhibit the respiration of isolated mitochondria, except under certain circumstances leading to oxaloacetate accumulation (29), or when much higher concentrations (of the order of 1 mM at pH 7.0) are used than are necessary to uncouple oxidative phosphorylation. It uncouples all three phosphorylation steps of the respiratory chain and thereby stimulates the respiration of ADP- or phosphate-deficient systems. Its action is correctly described as an "uncou-

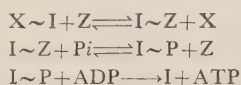
pler" of oxidative phosphorylation.

Oligomycin inhibits respiration like antimycin and blocks phosphorylation like dinitrophenol. However, unlike antimycin it has no effect on non-phosphorylating or uncoupled respiration. Thus, inhibition of respiration by oligomycin can be completely released by low concentrations of the uncoupling agent dinitrophenol (but not by arsenate). It is clear that oligomycin is primarily an inhibitor of oxidative phosphorylation and that inhibition of respiration is secondary to inhibition of phosphorylation.

In essence the formulation of the mechanism of oxidative phosphorylation favoured in this laboratory (30) states that the operation of the respiratory chain leads to the formation of a high-energy compound, which either directly or after transfer of the energy to other compounds, reacts with phosphate to form a $\sim P$ compound which reacts with ADP to form ATP. It is this last reaction which is responsible for the $ADP \rightleftharpoons ATP$ exchange reaction. Dinitrophenol reacts with a high-energy compound before the intervention of inorganic phosphate (23) and there is good reason to believe that the Mg^{2+} -stimulated ATPase involves an intermediate further removed from the respiratory chain than that involved in the action of dinitrophenol (31). This is supported by Chefurka's (32) finding that reduction of the respiratory chain inhibits the dinitrophenol-induced ATPase without having any effect on the Mg^{2+} -stimulated. The fact that oligomycin inhibits both ATPases without affecting the $ADP \rightleftharpoons ATP$ exchange reaction shows that the Mg^{2+} -stimulated ATPase cannot involve the terminal $\sim P$ compound, as previously supposed (31), but must involve an intermediate between this compound and the dinitrophenol-sensitive intermediate. Thus, there must be a minimum of three intermediates, which we shall write \sim_1 , \sim_2 and $\sim P$, respectively.



Arsenate presumably reacts instead of phosphate with the compound indicated here as \sim_2 , which cannot be a $\sim P$ compound. The fact that oligomycin inhibits both arsenate-induced respiration and the Mg^{2+} -stimulated ATPase can be explained on this basis only by assuming either (i) that it binds the \sim_2 compound or (ii) that it inhibits the formation of \sim_2 from both the \sim_1 compound and the $\sim P$ compound. For example, if we write the dinitrophenol-sensitive compound as $X \sim I$ and the \sim_2 compound by $I \sim Z$, we could have the reactions



If Z (which might be an enzyme) were inhibited by oligomycin the formation of $I \sim Z$ from both directions would be inhibited. To explain the inhibition of all phosphorylating steps in the respiratory chain, Z must be the same or similar for all three phosphorylating steps.

According to this explanation, non-phosphorylating preparations respire because the \sim_1 compound (or compound earlier in the sequence) can react with water. Thus, the effect of oligomycin is by-passed in the same way as by addition of dinitrophenol. The fact that addition of oligomycin to rat-liver mitochondria has exactly the same effect as omission of phosphate or ADP is good support for this view. Indeed, the degree of inhibition by oligomycin is related to the respiratory-control index by the equation.

$$\% \text{ inhibition} = 100 (r-1)/r$$

where r , the respiratory-control index, equals [rate in presence of ADP (or phosphate)]/[rate in absence of ADP (or phosphate)]. It is the general experience that this index decreases in the order of the substrates glutamate, succinate and ascorbate. So also do the measured P:O ratios in comparison with the expected values of 3, 2 and 1, respectively. It seems likely that mitochondrial preparations contain fragments which are particularly effective in oxidizing ascorbate by a non-phosphorylating pathway, less effective in oxidizing succinate and least effective

with glutamate.

Oligomycin is a very useful inhibitor. In addition to distinguishing between non-phosphorylating and phosphorylating pathways, it can distinguish between ATPases of mitochondrial and those of non-mitochondrial origin. For example, we have never been certain whether the ATPase, with pH optimum at 9.4, which is measured in rat-liver mitochondria in the absence of dinitrophenol was due to a mitochondrial system or to microsomal contamination. The fact that it is completely inhibited by oligomycin whereas microsomal (and myofibrillar) ATPases are not inhibited (Van Groningen, personal communication) shows that it is not of microsomal origin. Similarly, the active Mg^{2+} -stimulated ATPase of the Keilin and Hartree heart-muscle preparation appears to be of mitochondrial origin.

We have also found oligomycin useful in determining whether the response of a system to added phosphate is specifically related to oxidative phosphorylation or to a non-specific effect probably concerned with the physical properties of the mitochondria.

Finally, oligomycin should be a useful reagent to test whether the high-energy intermediates of oxidative phosphorylation can be directly utilized for energy-requiring reactions in the mitochondria, without having first to be converted to ATP (*cf.* Slater (33)).

SUMMARY

1. The findings of Lardy on the effects of oligomycin on oxidative phosphorylation and related reactions catalysed by rat-liver mitochondria were confirmed.

2. In addition, it was found that the fraction of the oxidation of succinate or glutamate which proceeded in the absence of ADP or inorganic phosphate was insensitive to oligomycin.

3. Phosphorylation associated with the oxidation of ascorbate by rat-heart sarcosomes was completely inhibited by oligomycin while the O_2 uptake was not affected.

4. In contrast to dinitrophenol, arsenate did not relieve the inhibition by oligomycin.

5. Oligomycin inhibits the ATPase of liver mitochondria measured at high pH's in the absence of dinitrophenol, as well as the dinitrophenol-stimulated ATPase under neutral and alkaline conditions. The Mg^{2+} -stimulated ATPase of non-phosphorylating mitochondrial fragments was also inhibited by oligomycin.

6. In contrast to antimycin, which is a respiratory inhibitor, and dinitrophenol, which is an "uncoupler" of oxidative phosphorylation, oligomycin is a true inhibitor of oxidative phosphorylation.

7. It is suggested that oligomycin combines with or prevents the formation of an energy-rich compound which lies between the dinitrophenol-sensitive energy-rich compound and the terminal phosphate compound in the sequence of reactions between the respiratory chain and ATP.

8. Oligomycin is a very useful reagent for distinguishing between non-phosphorylating and phosphorylating pathways, and between ATPases of mitochondrial and non-mitochondrial origin. It should also prove useful to test whether the high-energy intermediates of oxidative phosphorylation can be directly utilized for energy-requiring reactions in the mitochondria, without having first to be converted to ATP.

This work was supported in part by grant no. RG-6569 from the U.S. Public Health Service. We wish to thank Dr. W. C. Hülsmann and Mr. H. C. Hemker for fruitful discussions and Miss A. Searle and Mr. B. Winter for technical assistance.

REFERENCES

- (1) Warburg, O., "Schwermetalle als Wirkungsgruppen von Fermenten", Berlin (1948)
- (2) Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. (London) B*, **127**, 167 (1939)
- (3) Ahmad, K., Schneider, H. G., and Strong, F. M., *Arch. Biochem.*, **28**, 281 (1950)
- (4) Potter, V. R., and Reif, A. E., *J. Biol. Chem.*, **194**, 287 (1952)
- (5) Ernster, L., Jalling, O., Löw, H., and Lindberg, O., *Exptl. Cell Research*, suppl. **3**, 124 (1955)
- (6) Höllunger, G., *Acta Pharm. Toxicol.*, **11**, suppl. **1** (1955)
- (7) Lardy, H. A., Johnson, D., and McMurray, W. C., *Arch. Biochem. Biophys.*, **78**, 587 (1958)
- (8) Hogeboom, G. H., *Meth. Enzymol.*, **1**, 16 (1955)
- (9) Myers, D. K., and Slater, E. C., *Biochem. J.*, **67**, 558 (1957)
- (10) Cleland, K. W., and Slater, E. C., *Biochem. J.*, **53**, 547 (1953)
- (11) Keilin, D., and Hartree, E. F., *Biochem. J.*, **41**, 500 (1947)
- (12) Slater, E. C., *Biochem. J.*, **45**, 1 (1949)
- (13) Darrow, R. A., and Colowick, S. P., personal communication.
- (14) Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29**, 379 (1946)
- (15) Slater, E. C., *Biochem. J.*, **59**, 392 (1955)
- (16) Wadkins, C. L., and Lehninger, A. L., *J. Biol. Chem.*, **233**, 1589, 1599 (1958)
- (17) Greengard, P., Minnaert, K., Slater, E. C., and Betel, I., *Biochem. J.*, **73**, 637 (1959)
- (18) Slater, E. C., *Biochim. et Biophys. Acta*, **48**, 117 (1961)
- (19) Lardy, H. A., and Wellman, H., *J. Biol. Chem.*, **195**, 215 (1952)
- (20) Slater, E. C., and Hülsmann, W. C., in "Regulation of Cell Metabolism", Ciba Foundation Symposium (ed. G. E. W. Wolstenholme & C. M. O'Connor), p. 58, Churchill, London (1959)
- (21) Crane, R. K., and Lipmann, F., *J. Biol. Chem.*, **201**, 245 (1953)
- (22) Wadkins, C. L., *J. Biol. Chem.*, **235**, 3300 (1960)
- (23) Borst, P., and Slater, E. C., *Biochim. et Biophys. Acta*, **48**, 362 (1961)
- (24) Hemker, H. C., and Hülsmann, W. C., *Biochim. et Biophys. Acta*, **48**, 221 (1961)
- (25) Keilin, D., and Hartree, E. F., *Nature*, **176**, 200 (1955)
- (26) Maley, G. F., and Lardy, H. A., *J. Biol. Chem.*, **210**, 903 (1954)
- (27) Lehninger, A. L., Hassan, M., and Sudduth, H. C., *J. Biol. Chem.*, **210**, 911 (1954)
- (28) Lehninger, A. L., in *The Harvey Lectures*, **49** (1953-1954), Acad. Press Inc., New York, p. 176 (1955)
- (29) Slater, E. C., and Hülsmann, W. C., *Proc. Natl. Acad. Sci., U. S.*, in the press.
- (30) Slater, E. C., 'Proc. Intern. Symp. Enzyme Chem.', Tokyo & Kyoto, p. 288 (1957)
- (31) Purvis, J. L., and Slater, E. C., *Exptl. Cell Research*, **16**, 109 (1959)
- (32) Chefurka, W., *Can. J. Biochem. Physiol.*, **38**, 1195 (1960)
- (33) Slater, E. C., *Nature*, **172**, 975 (1953)
- (34) Estabrook, R. W., *Biochem. Biophys. Res. Commun.*, **4**, 89 (1961)
- (35) Lardy, H. A., and McMurray, W. C., *Federation Proc.*, **18**, 269 (1959)

Studies on the Metabolism of Rat-Ascites-Tumor with Nitrogen Mustard Sensitive and Resistant Strains

I. On the Aerobic and Anaerobic Glycolysis

By YOSHIKI MIURA,* NAKO OKAMOTO and HISASHI KATAYAMA

(From the Department of Biochemistry, Division of Health Care and Nursing,
Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, January 14, 1961)

The ascites hepatoma of the present study is prepared by conversion of the amino-azo-dye-induced hepatoma of the rat into the ascitic form by Yoshida and his collaborators (1, 2) and has been studied extensively in the past decade (3). Until the beginning of 1960, some twenty eight different strains of ascites hepatoma were isolated and found many strain-specific features.

Among many characteristic differences found within individual strains, the authors are interested in the facts that different responses were shown to nitrogen mustard derivatives by various strains of ascites hepatoma (3): A strain called AH 130 is usually preferred for the screening work for cancer chemotherapy because of its high trans-plantability as well as the benefit of ample free cells, beside generally small islands. Nitrogen mustard-N-oxide presented about 80 per cent complete cures in cases of animals bearing four-day-old tumors of AH 130. It was found in further experiments, however, that tumor animals of another ascites hepatoma, AH 7974, were surprisingly resistant to the same compound.

Besides, nitrogen mustard-N-oxide, other nitrogen mustard derivatives were tested on both ascites hepatomas, AH 130 and AH 7974, in order to find out the eventual differences in the response of both tumors to these compounds (3). In AH 130, all the compounds

could bring about a complete cure in almost 90 per cent of the animals tested as well as a prolongation of life of the remaining ones, while, in AH 7974, all the animals remained completely indifferent to all the compounds so far tested. It has thus been demonstrated that strain 7974 is completely refractory to all these alkylating agents that had significant inhibitory effects on the ascites hepatoma, AH 130.

Thus the comparative metabolic studies with strains AH 130 and AH 7974 should provide information on not only the refractory mechanism but also on the strain-specific metabolism of tumors.

In the present study, we have investigated the normal rate of aerobic and anaerobic glycolysis in both strains as well as the inhibitory action of alkylating agents, some synthetic seven-carbon-ring compounds* and other two antibiotics on this process.

MATERIALS AND METHODS

Animals—Albino rats weighing approximately 100 g. were used. Tumors of strains AH 130 or AH 7974 were transplanted intraperitoneally or subcutaneously in the case of solid tumor and the animals were sacrificed after 10 days of inoculation. Regenerating liver was obtained after 48 hours from rats which had been partially hepatectomized by the method of Higgins and Anderson (4). The livers and solid cancers were homogenized with 4 parts of a solution of 0.25M sucrose. In the experiments to study the

* Present address: Department of Physiological Chemistry, School of Medicine, Chiba University, Chiba.

* We are greatly indebted to Prof. Tetsuo Nozoe, the Department of Chemistry, Tohoku University for his generous supply of these compounds.

inhibitory action of anti-cancer agents, the whole ascites cells were used without homogenation, because of the accuracy of lactic acid determination whenever the low yield of the acid is obtained in the presence of inhibitors.

Isolation of Cell Nuclei—The isolation of the nuclei was carried out according to Hogeboom's method (5). To avoid contamination of the nuclei by erythrocytes, the blood cell layer in a centrifuge tube was mechanically removed by a small spatula.

Very few erythrocytes were observed by microscopic examination of the nuclear fraction and the contamination of mitochondria could not be demonstrated by the trichlorotetrazolium chloride test. Prior to the incubation, the nuclear fraction was washed with a solution of 0.25 *M* sucrose (without CaCl_2), separated by centrifugation at $550 \times g$ for 5 minutes, and then diluted with 10 parts of a solution of 0.25 *M* sucrose. The volume of the nuclei was noted.

Composition of Incubation Media—The following compositions of the incubation media were used for aerobic and anaerobic glycolysis.

Compounds	For aerobic glycolysis	For anaerobic glycolysis
	Final Conc.	Final Conc.
	<i>M</i>	<i>M</i>
Fructose diphosphate	0.01	0.01
Adenosine triphosphate	0.001	0.001
Diphosphopyridine nucleotide	0.00022	0.00022
Nicotinamide	0.04	0.04
Cytochrome c	0.00002	—
MgCl_2	0.0033	0.0033
Potassium phosphates (pH 7.4)	0.0033	—
Potassium fluoride	—	0.01
Potassium bicarbonate	—	0.02
Dibasic ammonium phosphate	—	0.02

The total volume of both incubation media was 5 ml. In the case of aerobic glycolysis, the incubation was carried out by shaking the cells under oxygen gas for 2 hours at 38°C in Erlenmeyer flasks. In the case of anaerobic glycolysis, the incubation was performed at 38°C for 2 hours in Thunberg tubes under vacuum. In the experiments concerning anti-cancer agents, the incubation time was limited within 30 minutes.

The determination of lactic acid—0.2–0.3 ml. aliquots were taken before incubation and at intervals of time, 0.5, 1.5 and 2 hours after the beginning of the incubation. The amount of lactic acid were determined by the method of Barker and Summerson (6). To simplify the condition, the effect of inhibitors were measured only after 30 minutes of incubation.

All inhibitors so far tested dissolved well in the incubation medium and thus all agents were introduced to the system from the very beginning of the reaction.

RESULTS

The amount of lactic acid produced during anaerobic glycolysis is shown in Fig. 1 and 2. In the homogenates of normal or regenerating livers, the amount of lactic acid

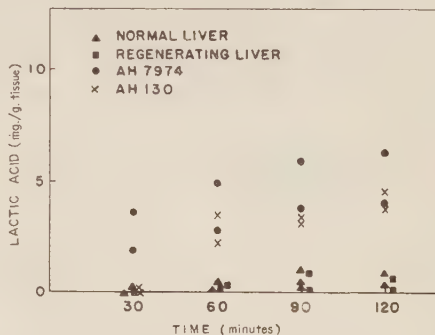


FIG. 1. The amount of lactic acid yielded by the homogenates under anaerobic condition. Other conditions are described in the text.

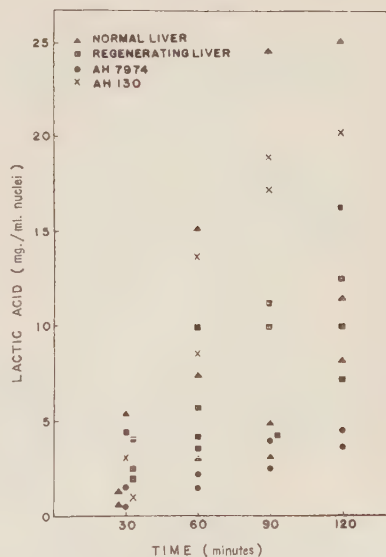


FIG. 2. The amount of lactic acid yielded by the cell-nuclei under anaerobic conditions. Other conditions are described in the text.

found in the incubation media did not increase appreciably during 2 hours period.

However in the homogenates of ascites hepatoma (AH 130 as well as AH 7974), lactic acid production increased with time. As shown in Fig. 2, the nuclei of AH 130 produced about 4 times more lactic acid than those of AH 7974. The nuclei of normal and regenerating liver produce much more lactic acid than the other parts of cells in terms of the wet-weight of tissue. The glycolytic potency of normal cell nuclei was, however, apparently lower than that of AH 130 nuclei but higher than that of AH 7974 nuclei. The similar tendency was observed in the case of aerobic glycolysis (Fig. 3 and 4). The sole

difference in aerobic condition was the lower production of lactic acid in normal and regenerating liver nuclei.

The inhibitory effects of some anticancer agents on glycolysis of normal liver homogenate and AH 130 cells are shown in Table I. It is, of course, difficult to compare the results of experiments with homogenates with those from free cancer cells because it should be account into the permeability factors of certain anticancer agents. However free liver cells obtained by the method of Kaltenbach (7) were not similar to free ascites cells from a metabolic point of view such as, for example, free liver cells showed, in fact, a good deal of oxygen consumption during an hour but it did not accompany the incorporation of P^{32} into nucleic acids. Furthermore, the homogenization of cancer tissues did not yield metabolically identical specimens in every instance. Thus, we prefer to use normal liver homogenates and free cancer cells as the materials for the studies of the effects of anticancer agents. Under anaerobic conditions, the lactic acid produced in normal liver homogenates or cancer cells was not so constant from experiment to experiment. For this reason, we have decided to express the values of lactic acid produced in the presence of anti-cancer agents as a per cent of that found in the uninhibited controls. In this way, the data could be reproducible whenever the absolute amount of lactic acid was different. In normal tissue, alkylating agents were equally inhibitory for both aerobic and anaerobic glycolysis, whereas, in cancer cells, the anaerobic glycolysis was usually more sensitive than the aerobic glycolysis to the action of alkylating agents at least when present at the concentration of $10^{-5} M$. Since the respiratory mechanism was also not sensitive to alkylating agents at a concentration of $10^{-5} M$, we were able to perform aerobically so-called "incorporation experiments."

The two antibiotics, Carzinophilin (8) and Mytomycin c (9) have been shown rather stimulatory effect on the aerobic glycolysis when present at a relatively low concentra-

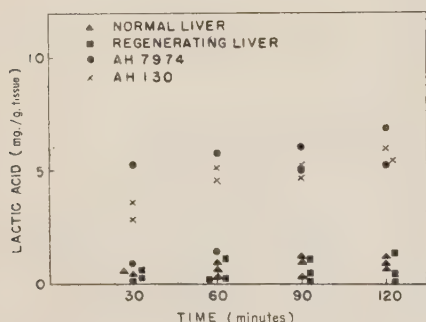


FIG. 3. The amount of lactic acid yielded by the homogenates under aerobic conditions. Other conditions are described in the text.

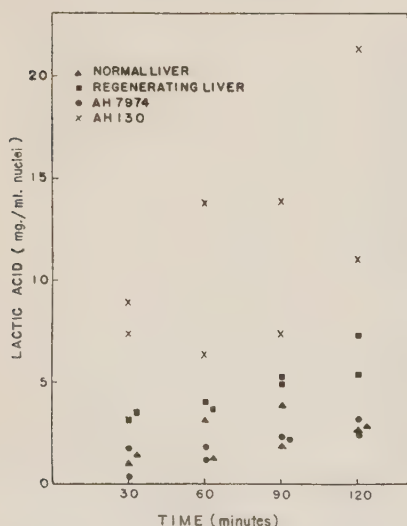


FIG. 4. The amount of lactic acid yielded by the cell-nuclei under aerobic conditions. Other conditions are described in the text.

TABLE I
*The Effects of Anticancer Agents on the Glycolysis of Normal Liver
 Homogenates and Ascites Hepatoma Cells*

Anticancer agents	Conc.	Glycolysis (Unihibited Control=100%)			
		Anaerobic		Aerobic	
		Normal liver homogenate	AH 130 cells	Normal liver homogenate	AH 130 cells
Nitrogen mustard	none	100%	100%	100%	100%
	$10^{-5} M$	63	75	53	108
	$10^{-3} M$	49	61	45	28
Nitrogen mustard N-oxide	$10^{-5} M$	106	89	100	98
	$10^{-3} M$	104	79	87	88
Thio-TEPA (Triethylenimino thiophosphoramide)	$10^{-5} M$	100	69	90	100
	$10^{-3} M$	94	39	89	42
Carzinophilin	20 u./ml.	81	126	116	103
	50 u./ml.	45	79	60	118
	500 u./ml.	54	55	57	38
Mitomycin c	5 μ g./ml.	98	114	101	113
	10 μ g./ml.	101	99	92	—
	500 μ g./ml.	—	—	109	—
	1000 μ g./ml.	86	41	145	—
1-Thia-3-azazulan-2-one	$10^{-7} M$	90	82	94	113
	$10^{-5} M$	90	62	78	58
2-Mercapto-1,3-diazazulene	$10^{-7} M$	100	114	94	93
	$10^{-5} M$	94	125	92	71
6-Amino-1,2,3-triazazulene	$10^{-7} M$	100	103	98	108
	$10^{-5} M$	94	92	73	108
2-Amino-1-azazulene	$10^{-7} M$	81	103	97	115
	$10^{-5} M$	75	71	70	106
Triazolo tropone	$10^{-7} M$	101	82	89	71
	$10^{-5} M$	90	82	82	60
1-Azazulan-2-one	$10^{-7} M$	63	90	110	100
	$10^{-5} M$	44	70	97	67

tion. The latter antibiotic had relatively little effect on the glycolysis of normal tissue as compared to Carzinophylin. In contrast to the effect of these recognized anti-cancer agents, certain seven-carbon-ring compounds have not been tested yet for the chemotherapy of experimental cancer, showed slight but aerobic and anaerobic glycolysis even at such low concentration as 10^{-7} molar. Among these compounds, 1-thia-3-azazulan-2-one was particularly interesting because of its strong inhibition not only of glycolysis but also of

respiration, ribonucleic acid synthesis and protein synthesis. The results of these experiments will be described in the successive reports.

DISCUSSION

Since the experiments of O. Warburg (10) in the late 1920's, it has been generally accepted that cancer cells show rather depressed respiration although aerobic as well as anaerobic glycolysis are enhanced in the energy yielding system of tumors. There are,

however, many cases which are exceptions to this theory that the glycolytic rate is high and the respiratory activity is low in normal tissue, and *vice versa*, in cancer cells (11).

Burk (12) using the Warburg manometric technique had already noted that cancer tissue demonstrated more potent glycolytic activity both under aerobic and anaerobic conditions than did normal tissue. In addition to these classic facts, we have demonstrated that cell nuclei in term of wet weight, showed more glycolytic activity than did the whole cells. This fact was more prominent in anaerobic glycolysis than in aerobic glycolysis. Although approximately equal amounts of lactic acid were produced in a given time with homogenates of both strains of ascites hepatoma, the glycolytic rates of cell nuclei varied according to the strain: *e.g.* high in AH 130 nuclei and low in AH 7974 nuclei.

LePage and Schneider (13) compared the glycolytic rate of rabbit liver nuclei with Flexner-Jobling carcinoma nuclei and demonstrated that cancer cell nuclei yielded about two times more lactic acid than did the normal liver nuclei. This result is not comparable to our experiments in which both the nuclei of normal liver and AH 7974 produced about same amount of lactic acid. Thus, it can not be stated as a general rule that the nuclei of cancer cells show higher glycolytic rate than those of normal liver.

The strain-specific metabolic activities of cancer cell nuclei were also observed in the case of alkaline phosphatase (14): The enzymatic activity of AH 130 nuclei was higher than that of AH 7974 nuclei. Other metabolic differences among different strains of ascites hepatoma will be shown in the succeeding reports.

The inhibitory actions of alkylating agents on glycolysis of cancer cells could not be the primary anti-cancer effect since the minimum growth-inhibiting effects of those agents were observed with much lower concentrations.

According to the report of the Research Institute of Kyowa Fermentation & Co. (19), the glycolysis of Ehrlich carcinoma cells was

very sensitive to Carzinophilin even with a dose of 200 units/ml. The aerobic glycolysis of Ehrlich carcinoma was also suppressed by the addition of 500 $\mu\text{g./ml.}$ of Mytomycin c. However Carzinophilin did not inhibit succinic dehydrogenase at a level of 5000 units/ml.

These reports are in good accord with our results. However, since the minimum effective doses of those anticancer agents for experimental cancer are about one tenth of the concentrations which were used in glycolytic inhibition, the inhibition of glycolysis by the two antibiotics might not be their main points of anti-cancerous effect.

SUMMARY

1. In the homogenate, ascites hepatoma produced more lactic acid than did normal liver under both aerobic and anaerobic conditions. There was, however, practically no differences between two strains of ascites hepatoma.

2. In the nuclear fraction, AH 130 showed a higher glycolytic rate than did AH 7974 and the rates of glycolysis in normal and regenerating liver nuclei were between the values found for AH 130 and AH 7974.

3. At the concentration of $10^{-5} M$, alkylating agents did not inhibit the aerobic glycolysis of the cancer cells. However the anaerobic glycolysis in cancer cells was more sensitive to the alkylating agents.

4. Two anti-cancer antibiotics did not inhibit the glycolysis *in vitro* at their minimum effective doses *in vivo*.

5. Among the azulenoide compounds, 1-thia-3-azazulan-2-one showed an inhibitory action for the glycolysis even at a concentration of $10^{-7} M$.

The work has been supported by grants from the Ministry of Education to Cancer Chemotherapy Research Group. We wish to thank the staff of the Sasaki Institute for their kind supply of AH strains-hepatoma.

REFERENCES

- (1) Yoshida, T., Sato, H., and Aruji, T., *Proc. Japan Acad.*, **27**, 485 (1951)

- (2) Sato, H., and Aruji, T., *Gann*, **43**, 254 (1952)
- (3) Yoshida, T., *Annal. N.Y. Acad. Sci.*, **63**, 852 (1956)
- (4) Higgins, G.M., and Anderson, R.M., *Arch. of Pathol. Lab. Med.*, **12**, 186 (1931)
- (5) Hogeboom, G.H., in S.P. Colowick & N.O. Kaplan's, "Methods in Enzymology", Academic Press, New York, Vol. **I**, p. 16 (1955)
- (6) Barker, S.B., and Summerson, W.H., *J. Biol. Chem.*, **138**, 535 (1941)
- (7) Kaltenbach, J.P., *Exptl. Cell Res.*, **2**, 568 (1954)
- (8) Hata, T., *J. Antibiotics* ser. A **7**, 107 (1954)
- (9) Wakai, S., Marumo, H., Tomioka, K., Shimizu, G., Kato, E., Kamada, H., Kudo, S., and Fujimoto, Y., *Antibiotics and Chemotherapy*, **8**, 228 (1958)
- (10) Warburg, O., "Metabolism of Tumors", translated by F. Dickens, Constable, London (1930)
- (11) Warburg, O., Weinhouse, S., Burk, D., and Shade, A.L., *Science*, **124**, 1267 (1956)
- (12) Burk, D., *Cold Spring Harbor Symp. Quant. Biol.*, **7**, 420 (1939)
- (13) LePage, G.A., and Schneider, W.C., *J. Biol. Chem.*, **176**, 1021 (1948)
- (14) Katayama, H., *Nissin Igaku*, **44**, 646 (1957)
- (15) Kamata, H., Wakaki, S., Kudo, S., Kumabe, K., and Kagawa, T., *J. Antibiotics*, ser. B. **11**, 2 (1958)

Studies on the Metabolism of Rat-Ascites-Tumor with Nitrogen Mustard Sensitive and Resistant Strains

II. On the Oxygen Consumption and Oxidative Phosphorylation

By YOSHIKI MIURA*, NAOKO OKAMOTO and MASAHISA GOTO

(From the Department of Biochemistry, Division of Health Care and Nursing,
Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, January 18, 1961)

The synthesis of high energy phosphate bonds as adenosine-triphosphate (ATP) is generally regarded as the probable mechanism mediating the energy necessary for normal cells as well as tumor cells. Although there is direct evidence that oxidative phosphorylations do occur in tumor (1-3), there are some difficulties in studying the coupled-phosphorylations in tumor homogenates. The principal trouble is the presence of large dephosphorylating activities which quickly destroy the ATP added or formed (4). This report presents results of a comparative study of oxidative phosphorylation by homogenates and mitochondria of tumor cells with those of normal as well as regenerating livers. The effect of various anticancer agents to the O_2 consumption as well as to the ratio of phosphorylation to oxygen consumption (*i.e.* P:O ratio) of normal mitochondria and tumor cells were also tested. It was found that some of these agents decreased specifically the P:O ratio of cancer cells.

MATERIALS AND METHODS

1. Determination of readily hydrolyzed phosphate esters formed by homogenates.

Homogenates were prepared with glass homogenizers by treating one part of tissue with four parts of 0.25 *M* sucrose solution. 0.2 ml. of homogenates of normal tissue or 1.0 ml. of homogenates of tumor tissue was added to the following incubation medium.

0.2 <i>M</i> Tris buffer (pH 7.4)	
in isotonic KCl	0.5 ml.
0.1 <i>M</i> $MgCl_2$	0.1 ml.
0.2 <i>M</i> α -Ketoglutarate	0.1 ml.
0.00025 <i>M</i> Cytochrome c	0.1 ml.
0.04 <i>M</i> Adenosine-5'-phosphate	0.4 ml.
0.1 <i>M</i> KH_2PO_4	0.2 ml.
0.25 <i>M</i> Sucrose	0.4 ml.
0.01 <i>M</i> KF (may be omitted	
in some cases)	0.1 ml.

The incubation was carried out at 37°C for 20 minutes. $\Delta 7P$ (inorganic phosphate released after 7 minutes boiling in 1 *N* HCl) was determined before and after incubation by means of Fiske-Subbarow's method.

2. The oxidative phosphorylation of mitochondria. Mitochondria were isolated from isotonic sucrose homogenates by the method described by Schneider (5). For the washing of mitochondria after the centrifugation at $8,500 \times g$, 0.44 *M* sucrose adjusted pH at 6 by addition of citrate buffer was used, instead of ordinary 0.25 *M* sucrose (6). The following incubation medium was adopted for the oxidative phosphorylation.

In a total volume 3 ml., it contains 0.05 *M* Histidine, 0.04 *M* KCl, 0.005 *M* $MgCl_2$, 0.01 *M* α -Ketoglutarate (or 0.02 *M* Succinate), 1.5×10^{-5} *M* Cytochrome c, 0.02 *M* phosphate buffer (pH 7.4), 0.008 *M* AMP, 0.025 *M* Glucose, 0.001 *M* DPN and 450 u. Hexokinase (Schwarz).

O_2 uptake was determined by the usual Warburg manometric method. After equilibration of 5 minutes, the incubation was carried out for 20 minutes at 30°C under air. The inorganic phosphate was determined by the method of Fiske-Subbarow before the incubation, after equilibration and after 20 minutes of incubation. The P:O ratio was calculated from the O_2 consumption and the amount of inorganic phosphate disappearance from the incubation medium.

* Present address: The Department of Physiological Chemistry, School of Medicine, Chiba University, Chiba.

RESULTS AND DISCUSSION

1. $\Delta 7P$ formed in homogenates.

Fig. 1 shows the amount of $\Delta 7P$ formed in homogenates during 20 minutes of incubation. The amount of energy rich phosphate compound yielded in the homogenates of normal livers was approximately ten times greater than when the incubation was carried out with tumor tissue. Under the conditions of these experiments, the addition of KF (7) did not change the amount of $\Delta 7P$ in tumor homogenates.

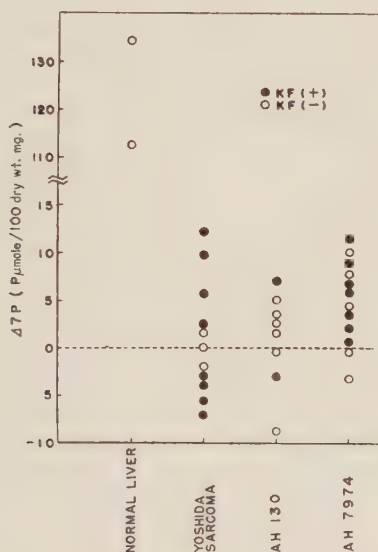


FIG. 1. The amount of $\Delta 7P$ yielded in the homogenates of normal liver and tumors. Other conditions are described in the text.

2. P:O ratios obtained in the oxidation of succinate and α -ketoglutarate by normal and neoplastic tissue in the Kielley's incubation medium.

The data on oxygen uptake of various mitochondria are given in Fig. 2. In general, the O_2 uptake of succinate oxidation was greater than that of α -ketoglutarate oxidation. This was especially evident in the case of normal and regenerating liver. The specific activities of α -ketoglutaric oxidase and succinoxidase in tumor and liver mitochondria were given in the data of R. K. Kielley (3). Expressed as μ mole per hour per mil-

ligram of nitrogen, the activities for liver mitochondria are as follows: α -Ketoglutaric oxidase, 19; succinoxidase, 58; for tumor mitochondria, these values are 18 and 38, respectively. The specific activities of α -keto-

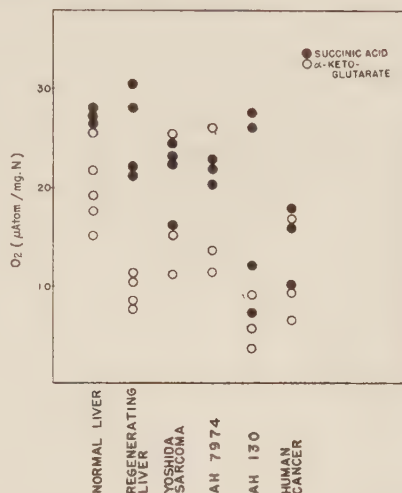


FIG. 2. Oxygen consumption of various mitochondria.

glutaric oxidase in liver and tumor mitochondria were nearly the same, whereas the specific activity of succinoxidase of tumor mitochondria was only 65 per cent that of the normal liver mitochondria.

However, in the present experiment the differences between the O_2 uptake resulting

TABLE I
Weight of Mitochondrial Fraction and
Nitrogen Content

	Weight of mitochondrial fraction (mg.) per g. of tissue	Total N (μ g.) per mg. mitochondria
Normal liver (8 cases)	149 mg.	23 μ g.
Regenerating liver (6 cases)	153	16
Yoshida sarcoma (4 cases)	99	7
AH 130 (3 cases)	95	8
AH 1974 (6 cases)	87	11

from the oxidation of succinate with normal mitochondria and tumor mitochondria were not so great. It may be possible to say that

the O_2 uptake of normal and neoplastic mitochondria are nearly the same in terms of mitochondrial nitrogen. But as shown in Table I, the nitrogen content of mitochondria as well as weight of mitochondrial fraction in a tumor cells are far less than in the normal and therefore the O_2 uptakes of tumor mitochondria in terms of an equivalent weight of tissue would be significantly lower than those of normal mitochondria. The uptakes of inorganic phosphate by normal and neoplastic mitochondria are shown in Fig. 3. The

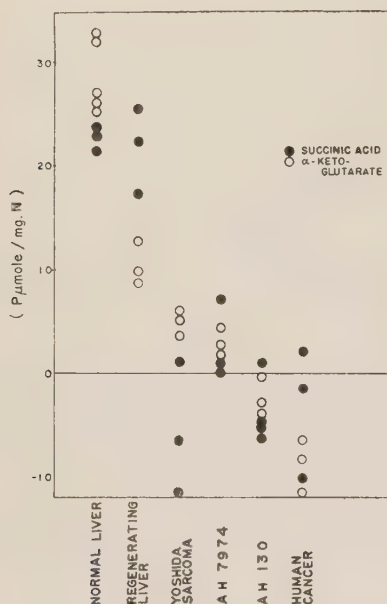


FIG. 3. Phosphate uptake of various mitochondria.

disappearance of inorganic phosphate from the medium was the greatest in the case of normal liver mitochondria while the lower uptake of inorganic phosphate was observed in the case of tumor mitochondria. The phosphate uptakes of normal and regenerating liver mitochondria resulting from the oxidation of succinate were approximately at the similar level. However, the uptake of inorganic phosphate in regenerating liver mitochondria resulting from the oxidation of α -ketoglutarate was far less than that in normal liver mitochondria. There is a similar tendency in respect to the O_2 consumption.

Thus, as shown in Fig. 4, the P:O ratios of regenerating liver mitochondria resulting from the oxidation of α -ketoglutarate were around 1.0 while, P:O ratios between 1.0 and 2.0 were obtained in the case of normal mitochondria.

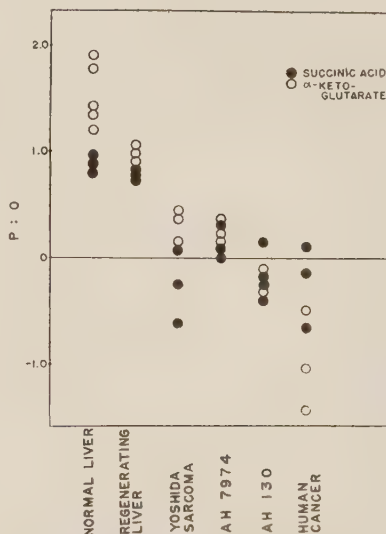


FIG. 4. The P:O ratios obtained with the oxidation of succinate and α -ketoglutarate by various mitochondria.

Negative values for phosphate uptakes were occasionally obtained with the mitochondria isolated from Yoshida Sarcoma, AH 130 and human cancers but not from AH 7974.

Weinhouse (8) found that the oxidation of many Krebs-cycle intermediates in tumor homogenates was favored by the addition of DPN. Kielley and Kielley (9) also reported that the addition of DPN significantly increased the rate of phosphorylation during the oxidation of α -ketoglutarate and glutamate.

In our experiments, DPN was already added into the incubation medium. Moreover the phosphate uptake of tumor mitochondria by the oxidation of succinate in which DPN is not involved as the hydrogen transfer system, showed also some negative values. Thus the deficiency of DPN might not be the real cause of the insufficient phosphorylation activity of tumor.

TABLE II

The Effects of Some Anticancer Agents on the O₂ Consumption and the P:O Ratios of Normal Liver Mitochondria and AH 130 Cells

Anticancer agents	Conc.	O ₂ consumption		P:O	
		Normal mitochondria	AH 130 cells	Normal mitochondria	AH 130 cells
Nitrogen mustard	none	100%	100%	1.17	0.37
	10 ⁻⁵ M	111	99	0.11	0.39
	10 ⁻³ M	100	111	0.0	0.17
Nitrogen mustard N-oxide	none	100	100	1.12	0.17
	10 ⁻⁵ M	117	95	0.32	0.16
	10 ⁻³ M	143	85	0.40	0.23
Thio-TEPA	none	100	100	1.28	0.40
	10 ⁻⁵ M	96	93	0.68	0.35
	10 ⁻³ M	83	53	0.70	0.38
Carzinophilin	none	100	100	1.14	0.19
	20 u/ml.	93	80	0.85	0.09
	50 u/ml.	96	97	0.80	0.08
	500 u/ml.	104	61	0.07	0.08
	1000 u/ml.	51	36	0.07	0.03
Mitomycin c	none	100	100	0.90	0.33
	5 µg./ml.	94	103	0.21	0.41
	10 µg./ml.	96	82	—	0.38
	100 µg./ml.	94	79	0.20	0.26
	1000 µg./ml.	89	33	0.13	0.32
1-Thia-3-aza azulan-2-one	none	100	100	0.80	0.22
	10 ⁻⁷ M	71	72	0.35	0.18
	10 ⁻⁵ M	64	75	0.23	0.16
2-Mercapto-1, 3- diazazulene	none	100	100	0.69	0.76
	10 ⁻⁷ M	90	80	0.42	0.57
	10 ⁻⁵ M	87	95	0.05	0.80
6-Amino-1, 2, 3- triazazulene	none	100	100	1.04	0.63
	10 ⁻⁷ M	108	109	0.56	0.54
	10 ⁻⁵ M	85	91	0.29	0.35
2-Amino-1, 3- diazazulene	none	100	100	0.85	0.52
	10 ⁻⁷ M	101	92	0.81	0.06
	10 ⁻⁵ M	98	89	0.21	0.06
Triazolotropone	none	100	100	0.69	0.35
	10 ⁻⁷ M	81	76	0.10	0.35
	10 ⁻⁵ M	74	69	0.06	0.38
1-Azazulene-2-one	none	100	100	0.75	0.30
	10 ⁻⁷ M	73	62	0.59	0.47
	10 ⁻⁵ M	84	20	0.23	0.30

In order to prevent the action of ATP-ase, after the isolation of mitochondria and before the incubation, we kept the pH at 6 a condition which is not favorable for the action of ATP-ase. From our own experiments on the determination of $\Delta 7P$ and from the experimental data of Kielley (3), it

may be seen that the addition of fluoride is not always effective in the inhibition ATP-ase activities. Therefore we could not assume that the negative values of P:O ratios observed in the case of tumor mitochondria were due to the lack of KF in the incubation medium. Among the investigations in this

field, there exist conflicting results: Kielley (3) reported that the P:O ratios obtained during the oxidation of α -ketoglutarate with tumor mitochondria in mice were up to 2.2, and those obtained during the oxidation of succinate, up to 1.3; Rosenberg (10) reported values of up to 1.7 for the P:O ratios with mitochondria isolated from rat-ascites-hepatoma. In contrast with such higher ratios, Yagi and Khouvine (11) reported lower values, around 0.3, for the P:O ratios obtained by the oxidation of succinate with a typical rat-epithelioma.

Yet, we believe that the lower P:O ratios obtained by tumor mitochondria were due to the phosphatase activity activated by the destruction of the cells since the P:O ratios of succinate oxidation with the mitochondria isolated from AH 130 were significantly lower those obtained with the whole cells of AH 130. The former values were almost below zero whereas the latter, though still lower than those of normal mitochondria, showed no negative values in a dozen of trials (Fig. 5).

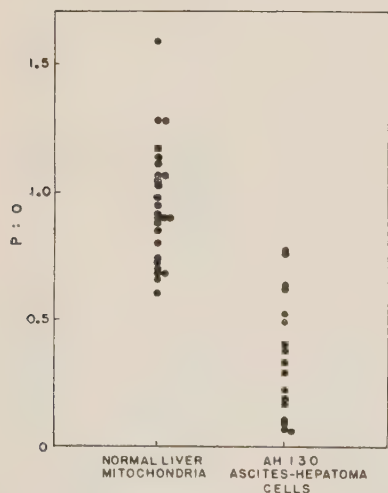


FIG. 5. Comparison of the P:O ratios of succinate oxidation obtained with normal liver mitochondria and AH 130 ascites-hepatoma cells.

The effects of some anticancer agents like nitrogen-mustard, nitrogen-mustard-N-oxide, Thio-TEPA, Carzinophilin, Mitomycin c and azulenoide compounds on the O_2

consumption as well as on the P:O ratios are given in Table II. At the minimum effective dose for a therapeutic effect *in vivo*, the addition of anticancer agents did not inhibit significantly the oxygen consumption of normal liver mitochondria or AH 130 cells. However, among the azulenoide compounds, the addition of 1-thia-3-azazulan-2-one at a concentration of 10^{-7} M inhibits oxygen consumption to the extent of 30 per cent.

In comparison with the effects of anticancer agents on the O_2 consumption, the influence of those agents on the P:O ratios were significantly greater than we expected: The addition of alkylating agents and antibiotics to the normal mitochondria lowered the efficiency of the phosphorylation, whereas the addition of these agents to the neoplastic mitochondria did not show such significant decrease of the P:O ratio. This means that the originally low values of the P:O ratio in neoplastic tissue were not affected so much by the addition of anticancer agent.

SUMMARY

1. The amounts of $\Delta 7P$ produced by the oxidation of α -ketoglutarate and succinate in the homogenates of normal liver and various tumors were determined. It was found that the production of $\Delta 7P$ in normal homogenates was higher than in the tumor homogenates. The addition of KF did not increase the lower production of $\Delta 7P$ in tumor homogenates.

2. The oxygen consumption of the various mitochondria did not show any remarkable differences between normal and neoplastic mitochondria, especially in the case of the oxidation of succinate. However, as the uptakes of inorganic phosphate were so weak in tumor mitochondria, the P:O ratios in tumor showed very low values compared to the normal tissue.

3. The P:O ratios of AH 130 mitochondria were lower than those of AH 7974.

4. The effects of some anticancer agents on the O_2 uptake and the P:O ratios of normal liver mitochondria and AH 130 cells

were tested. It was found that the addition of many anticancer agents apparently decreased the P:O ratios of normal mitochondria even when present in relatively low concentration.

The work has been supported by grants from the Ministry of Education to Cancer Chemotherapy Research Group. The authors are indebted to the staffs of the Sasaki Institute for their kind cooperation in supplying AH strains of ascites hepatoma.

REFERENCES

- (1) Potter, V. R., *J. Cell. and Comp. Physiol.*, **26**, 87 (1945)
- (2) Clowes, G. H. A., and Keltch, A. K., *Proc. Soc. Exp. Biol. and Med.*, **77**, 369 (1951)
- (3) Kielley, R. K., *Cancer Res.*, **12**, 124 (1952)
- (4) Potter, V. R., *Cancer Res.*, **11**, 565 (1951)
- (5) Schneider, W. C., *J. Biol. Chem.*, **176**, 259 (1948)
- (6) Witter, R. F., Watson, M. L., and Cottone, M. A., *J. Biophys. Biochem. Cytol.*, **1**, 127 (1955)
- (7) Potter, V. R., and Lyle, G. G., *Cancer Res.*, **11**, 255 (1951)
- (8) Weinhouse, S., Millington, R. H., and Wenner, C. E., *J. Am. Chem. Soc.*, **72**, 4332 (1950)
- (9) Kielley, W. W., and Kielley, R. K., *J. Biol. Chem.*, **191**, 485 (1951)
- (10) Andrejew, A., and Rosenberg, A. J., *C. R. Soc. Biol.*, **150**, 1855 (1956)
- (11) Yagi, K., Matsuoka, Y., and Khouvine, Y., *Bull. Soc. Chim. Biol.*, **39**, 1243 (1957)

Studies on the Metabolism of Rat-Ascites-Tumor with Nitrogen Mustard Sensitive and Resistant Strains

III. On the Incorporation of Labelled Amino Acids into Protein *in vitro*

By YOSHIAKI MIURA, KENHIKO MOMOSE and AKIKO MORIYAMA

(From the Department of Physiological Chemistry, Chiba University,
School of Medicine, Chiba)

(Received for publication, March 8, 1961)

The cytotoxic alkylating agents, such as nitrogen-mustard, nitrogen-mustard-N-oxide and Thio-TEPA (Triethylenimino thiophosphoramidate) are known to react *in vitro* under mild conditions with a wide variety of compounds, such as water, anions, bases, amino acids, protein and nucleic acids (1). Although the mode of action of these alkylating agents on the cell is not known, it has been suggested that the chemical sites of reaction may be ionizable acid groups, either carboxyl groups of amino acids and protein or phosphate groups of nucleic acids, especially deoxyribonucleic acids (2).

A study of the effects of nitrogen-mustard on the *in vitro* incorporation of labelled amino acids into the protein of two strains of rat-ascites hepatoma, AH 130 (nitrogen-mustard sensitive strain) and AH 7974 (nitrogen-mustard resistant strain) has been carried out to ascertain whether the refractory character of AH 7974 is due to the change of the biosynthetic process of protein.

Nitrogen-mustards used in this study were kindly provided by Dr. Y. Sakurai of the Iatrochemical Research Institute.

MATERIALS AND METHODS

Universally labelled C^{14} -amino acids were obtained from the protein hydrolysates of algae and provided to us through the courtesy of Dr. B. Maruo of the Institute of Applied Microbiology.

Cell Preparations—AH strain of ascites-hepatoma cells were grown for 8–10 days in the abdominal cavity of rats. The animals were killed by decapitation, and the ascitic fluid was collected by pipette.

The cells were washed two times with either ice-cold, calcium-free Krebs-Ringer phosphate or 0.25 *M* sucrose.

To remove blood elements, quick washing with ice-cold tap-water was occasionally applied.

Incubation Methods—The composition of the medium for the incubation of the whole cells is given in μ moles: $MgCl_2$ 105, K_2HPO_4 50, KCl 1050, α -ketoglutarate 45, Cytochrome c 0.3, adenosine triphosphate (ATP) potassium salt 15, folic acid 2.5. The liquid volume is 1 ml. To this synthetic medium, 4 ml. of ascitic fluid with hepatoma cells (approximately 10 mg. dry weight cells per ml.) and 1 μ c of radioactive amino acids were added and the pH was adjusted at 7.4. Thus the total volume of the 5 ml. incubation medium was kept at 37°C for one hour under oxygen gas.

The incubation medium for experiments with the microsomal fraction of hepatoma is given in μ moles: potassium phosphate buffer (pH 7.6) 50, KCl 30, $MgCl_2$ 5, ATP 8, guanosine triphosphate (GTP) 0.5, creatine phosphate 14.3. The total volume is 1 ml. To this medium, 0.5 mg. of creatine kinase, approximately 1.5 mg. of microsomes 3 mg. of pH 5-enzymes, and 1 μ c of C^{14} -amino acids were dissolved and kept at 37°C for 30 minutes under air.

The incubation medium for the combination of S-RNA and C^{14} -amino acids (3) is given in μ moles: potassium phosphate buffer (pH 7.8) 50, KCl 60, $MgCl_2$ 30 and ATP 200. In addition to these compounds, 2 μ c of C^{14} -all-labelled amino acids and approximately 1.3 mg. of pH 5-enzyme fraction of hepatoma cells were mixed in the total 2 ml. of medium. The incubation was carried out at 37°C for 10 minutes.

Preparations of Subcellular Fractions—The microsome and pH 5-enzymes fractions were obtained from the hepatoma cells with method of Takanami (4).

Assay of Incorporated Radioactivity—After the incubation period and just before the reaction was stopped

by adding an equal volume of 10% perchloric acid (PCA), a mixture of non-labelled amino acids was added as a carrier to remove non-incorporated radioactive amino acids. Since labelled amino acids incorporate into the fraction of phosphatide-peptide which may be considered as the protein fraction of the ordinary Schneider's fractionation method, all the experiments except that of the extraction of S-RNA-amino acids complex were carried out according to Huggins and Cohn's fractionation method (5). The extraction of the S-RNA-amino acids complex used the method described by Hoagland *et al.* (3).

The proteins were suspended in water, plated on aluminum discs and counted by gas flow counter. The radioactivity was corrected for selfabsorption and expressed by the specific activity (reference weight of 10 mg. (6)). In the case of S-RNA-amino acids, the specific activity was expressed c.p.m. per microgramm of ribonucleic acid. In this case, the radioactivities were calculated for samples of infinite thinness.

RESULTS

Experiments with Whole Cells—The initial experiments with whole hepatoma cells were carried out to assess the influence of nitrogen-mustard on the incorporation of radioactivity from C^{14} -amino acids. The concentration of nitrogen-mustard used in this experiments was $2 \times 10^{-5} M$, which did not inhibit the respiration of the cells in the previous experiments.

It was found, as may be seen in typical results given in Table I, that the inhibition of the incorporation of amino acids into protein by adding nitrogen-mustard was far greater in the sensitive strain (AH 130) than in the resistant strain (AH 7974). In the case of the AH 130 strain, the addition of $2 \times 10^{-5} M$ of nitrogen-mustard resulted in a 46 per cent decrease in the amino acid incorporation in the protein fraction as compared to the control cells. Whereas, in the AH 7974 strain, as high as 80 per cent incorporation was observed with the addition of the same concentration of nitrogen-mustard.

The removal of folic acid or ATP showed slightly increased effects of nitrogen-mustard in the case of AH 130 but not in the case of AH 7974.

A correlation between the incorporation rate of amino acids into protein and that into lipid fraction was observed both with AH 130 and with AH 7974. The weight of phosphatido-peptide was usually too small for accurate calculation of the specific activities of this fraction.

Experiments with Subcellular Fractions—Table II shows the results of three typical experiments with microsomal proteins.

TABLE I

The Effect of Nitrogen-mustard on the Incorporation of C^{14} -all-labelled Amino Acids into Protein, Lipid and Phosphatido-peptide Fractions of Hepatoma Cells

Hepatoma	Condition	Nitrogen mustard	Radioactivity found in		
			Protein c.p.m./mg.	Lipid c.p.m./mg.	Phosphatido-peptide c.p.m./mg.
AH 130	Complete system	none	866 (100%)	149 (100%)	25
	Complete system	$2 \times 10^{-5} M$	463 (54%)	78 (53%)	23
	Complete system —folic acid	$2 \times 10^{-5} M$	392 (45%)	70 (47%)	18
	Complete system —ATP	$2 \times 10^{-5} M$	371 (43%)	69 (47%)	32
AH 7974	Complete system	none	596 (100%)	172 (100%)	32
	Complete system	$2 \times 10^{-5} M$	484 (80%)	136 (79%)	106
	Complete system —folic acid	$2 \times 10^{-5} M$	527 (89%)	146 (86%)	44
	Complete system —ATP	$2 \times 10^{-5} M$	534 (89%)	135 (79%)	82

TABLE II

Effect of Nitrogen-mustard on the Incorporation of C¹⁴-all-labelled Amino Acids into Microsomal Protein, Lipid and Phosphatido-peptide Fraction of Hepatoma

Hepatoma strain	Experiment number	Concentration of nitrogen mustard	Radioactivity found in		
			Protein c.p.m./mg.	Lipid c.p.m./mg.	Phosphatido-peptide c.p.m./mg.
AH 130	Exp. 1	none	127 (100%)	147 (100%)	3
		10 ⁻⁵ M	114 (90%)	— —	2
	Exp. 2	none	61 (100%)	152 (100%)	15
		10 ⁻⁴ M	— —	149 (98%)	16
	Exp. 3	none	59 (100%)	83 (100%)	1
		10 ⁻⁵ M	48 (81%)	— —	—
AH 7974	Exp. 1	none	77 (100%)	549 (100%)	—
		10 ⁻⁵ M	63 (82%)	503 (92%)	—
	Exp. 2	none	149 (100%)	77 (100%)	20
		10 ⁻⁴ M	106 (72%)	30 (43%)	17
	Exp. 3	none	75 (100%)	423 (100%)	1
		10 ⁻⁵ M	60 (80%)	393 (93%)	5
		10 ⁻⁴ M	57 (76%)	— —	—

In this table, the use of ten times more concentrated nitrogen-mustard does not show any significant increase in inhibition. Furthermore the nitrogen-mustard sensitive strain of hepatoma is not always more sensitive than the resistant strain from the view point of the inhibitory effects of nitrogen-mustard on the incorporation of the labelled compounds into protein and lipid fractions.

The data presented in Table II demonstrates that if the cell-membranes were removed prior to incubation, the effects of nitrogen-mustard were almost equal for both AH 130 and AH 7974. This might be related to the differing rates of entry of the agent into the cells or to different affinities to a receptor system on the cell-membranes. Furthermore, they show that there was no definite correlation between the concentration of nitrogen-mustard and the rate of inhibition of amino acid incorporation into protein. For example, in AH 7974, the addition of nitrogen-mustard at the concentration of 10⁻⁵ M gave a 18–20 per cent inhibition of incorporation into the proteins, whereas the inhibition was

only 24–28 per cent with the concentration of 10⁻⁴ M.

In contrast to the experiments with whole cells, there were no correlations between the incorporation rate into protein and that into lipid.

Since incorporation of C¹⁴-amino acids into phosphatido-peptide fraction in this system is very little, accurate results could not be obtained in the present experiment.

Experiments of the Combination of S-RNA and Amino Acids—As the result obtained with microsomal protein shows slight but steady inhibition of the amino acids incorporation into protein with the addition of nitrogen-mustard, it should be interesting if the site of action of nitrogen-mustard is elucidated in the course of protein synthesis. Thus, in Table III, the inhibition of the combination of soluble ribonucleic acids with C¹⁴-amino acids by nitrogen-mustard is demonstrated.

Every time, the actual count of the C¹⁴-amino acids-S-RNA complex was about several hundred counts per minute and the combination of C¹⁴-amino acids and soluble-

RNA was almost complete in the initial five minutes. However, in the presence of $10^{-4} M$ nitrogen-mustard, the combination is not

TABLE III

The Effect of Nitrogen-mustard on the Combination of S-RNA and C^{14} -all-labelled Amino Acids by pH 5 Enzymes of Hepatoma

	Nitrogen mustard	Combination c.p.m./ μ g. RNA	Rate %
AH 130	none	2.2	100
	$10^{-4} M$	1.7	74
AH 7974	none	8.8	100
	$10^{-4} M$	6.6	74

completed as shown in Fig. 1.

With the use of $10^{-4} M$ nitrogen-mustard, the two enzyme systems derived from AH 130 cells and AH 7974 cells both gave a 25 per

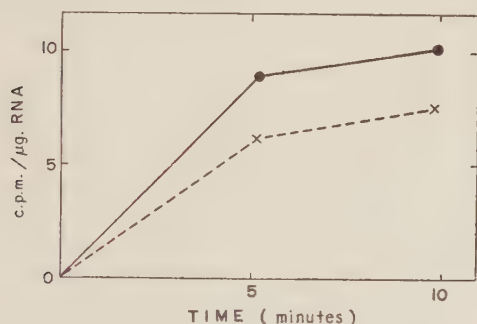


FIG. 1. Effects of $10^{-4} M$ nitrogen-mustard on the combination of C^{14} -amino acids with soluble ribonucleic acids of AH 7974 hepatoma.

cent inhibition of the combination. The 25 per cent inhibition corresponds well with the results obtained in the experiment using microsomal protein.

DISCUSSION

From the results obtained in this series of experiments, it was shown that the destruction of the cell-membranes lowered the high susceptibility of AH 130 cells to that of nitrogen-mustard. This fact may not lead to the conclusion that the permeability of the cell-membrane is responsible for the characteristic feature of the nitrogen-mustard resistant

strain since the destruction of the cell-membranes of AH 130 showed a lower inhibition rate by the addition of nitrogen-mustard: at the concentration of $2 \times 10^{-5} M$ nitrogen-mustard, the incorporation into protein of labelled amino acids by whole AH 130 cells was 54 per cent, whereas at the concentration of $10^{-5} M$ nitrogen-mustard the inhibition of the incorporation into microsomal protein by the cell-free system was 81 per cent. We would like to postulate the existence of some specifically sensitive receptor system in AH 130 cells rather than the case of permeability. Once the cells were destroyed, no significant differences in inhibition between the AH 130 and AH 7974 strains were observed. The inhibition of 20–30 per cent by adding 10^{-5} – $10^{-4} M$ of nitrogen-mustard might come from a disturbance of the combination of C^{14} -amino acids with soluble ribonucleic acids, as shown in Table III. The ceiling effect at 10^{-5} – $10^{-4} M$ nitrogen-mustard may be interpreted as following. At these concentrations the nitrogen-mustard saturates almost all the sites where the combination of soluble ribonucleic acid with amino acids occurs, and so greater concentrations do not further increase the inhibition.

Thus the cause of the sensitivity to the nitrogen-mustard of AH 130 cells might be considered to be the existence of the specific receptor system of AH 130 cells.

Coles, Johnstone and Quastel (7) studied the effect of a number of alkylating agents on the *in vitro* incorporation of glycine- C^{14} into the ethanol/acetone-insoluble fraction of Ehrlich ascites carcinoma cells. With the exception of a compound, all alkylating agents so far tested (including nitrogen-mustard) inhibit this incorporation in varying degrees, with little or no effect on the respiration of the cells.

In their experiment, the inhibitory effect of an alkylating agent on the incorporation of glycine- C^{14} into the proteins of tissues increases with time of exposure of the cells to the agent.

Different tumors have different susceptibilities to alkylating agents as far as inhibi-

tion of glycine-1-C¹⁴ incorporation is concerned.

These results were almost similar to our results, except some newer alkylating agents like ethyl methane sulfonate were used at higher concentrations and showed consequently stronger inhibitions.

Their most interesting result is the fact that they have postulated a hypothetical receptor system in the susceptible tissue: Studies of the effects of nitrogen-mustard show that if inhibition of the process of glycine incorporation in Ehrlich ascites proteins is proportional to the amount of alkylating agents adsorbed on a cell receptor, the adsorption follows the Langmuir adsorption isotherm.

According to our experiments, AH 130 cells are more susceptible than AH 7974 cells. However if we destroyed the cell-membranes, the difference in susceptibility disappeared at once. Thus, if we postulate some specific receptor system on the surface of AH 130 cells, the singular susceptibility of AH cells might be partially interpreted by this hypothesis.

At present the nature of receptor systems for alkylating agents which may exist on the surface of susceptible cells cannot be stated. However, Roberts and Warwick (8) studied the metabolism of ethyl methane sulfonate in the rat and showed that this agent is conjugated with cysteine to form N-acetyl-S-ethylcysteine, which is then excreted in the urine. Thus, the combination with SH compounds may be an important step in considering the mode of action of certain alkylating agents.

SUMMARY

1. The incorporation *in vitro* of C¹⁴-amino acids into protein of rat-ascites hepatoma cells

was inhibited by the addition of 10⁻⁵ M nitrogen-mustard to the incubation medium. AH 130 hepatoma cells were more susceptible than AH 7974 hepatoma cells.

2. With the homogenates of hepatoma cells, the difference in susceptibility of the hepatoma strain disappeared and the susceptibility of the hepatoma strain disappeared and the susceptibility of the AH 130 strain was slightly decreased.

3. The combination of soluble ribonucleic acid with C¹⁴-amino acids was inhibited by the addition of nitrogen-mustard.

This might be the cause of the inhibitory action of nitrogen-mustard during the course of protein biosynthesis, but the authors have postulated some specific receptor system on the surface of the susceptible hepatoma cells.

We gratefully acknowledge financial assistance by the Ministry of Education. We are also most grateful to Professor T. Yoshida and the staff of the Sasaki Institute for their valuable advice.

REFERENCES

- (1) Ross, W. C. J., *Ann. New York Acad. Sci.*, **68**, 669 (1958)
- (2) Stacey, K. A., Cobb, M., Cousens, S. F., and Alexander, P., *Ann. New York Acad. Sci.*, **68**, 682 (1958)
- (3) Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I., and Zamecnik, P. C., *J. Biol. Chem.*, **231**, 241 (1958)
- (4) Takanami, M., *Biochim. et Biophys. Acta*, **39**, 318 (1960)
- (5) Huggins, C. G. and Cohn, D. V., *J. Biol. Chem.*, **234**, 257 (1959)
- (6) Hendler, R. W., *Science*, **130**, 772 (1959)
- (7) Coles, N. W., Johnstone, R. M., and Quastel, J. H., *Cancer Res.* **20**, 1523 (1960)
- (8) Roberts, J. J., and Warwick, G. P., *Biochem. Pharmacol.*, **1**, 60 (1958)

Studies on the Metabolism of Rat-Ascites-Tumors with Nitrogen Mustard Sensitive and Resistant Strains

IV. On the Distribution of Various Enzymes in the Cells of Normal Liver, Regenerating Liver and Hepatoma

By YOSHIKI MIURA, SHIN FURIYA, YOSHIRO ASO, ATSUSHI IMADOME,
AKIRA NISHIMURA and OSAMU URUSHIBARA

(From the Department of Physiological Chemistry, Chiba University,
School of Medicine, Chiba)

(Received for publication, March 13, 1961)

Since the development of the subcellular fractionation method, the fact that many enzymes are unequally distributed in the cells has now been firmly established by several investigators. Among them, C. de Duve and his group (1) have already reported intracellular distribution patterns of enzymes in normal rat liver tissues.

This paper is a comparative study on the intracellular distribution patterns of some enzymes with normal livers, regenerating livers and two strains of rat-ascites-hepatoma. These patterns were different for normal and regenerating livers as well as for two strains of DAB-hepatoma. However, the greatest difference was generally observed between normal liver and hepatoma even after taking account of the fundamental difference in the mass of the nuclei or mitochondria of those two tissues.

EXPERIMENTAL

The experiments were performed on the livers of albino rats weighing 100–120 g. and on rat-ascites-hepatoma cells (AH 130 strain and AH 7974 strain) implanted in the abdominal cavity of albino rats. The hepatoma cells were harvested after 10–12 days implantation and homogenized with 0.25 *M* sucrose and made 20% homogenates. Regenerating livers were made by the method of Higgins and Anderson (2) and used 48 hours after the surgical removal of a part of the livers. The subcellular fraction was carried out by the method described by Hogeboom (3). Nucleal, mitochondrial, microsomal and supernatant fractions were obtained by the differential centrifugation.

Enzyme Assays

Acid Phosphatase—The activity of this enzyme was measured by the method described by Ohmori and Fujita (4). To 1.0 ml. of substrate solution containing 0.001 *M* *p*-nitrophenol phosphate and 0.1 *M* acetate buffer (pH 5.0) was added 1.0 ml. of enzyme solution (in the form of homogenates or of separate subcellular fractions). The total volume of 2.0 ml. was incubated at 37°C for 30 minutes. The reaction was stopped by adding 2.0 ml. of 10% trichloroacetic acid. After centrifugation, 2.0 ml. of the supernatant solution was added to the same volume of saturated Na₂CO₃ solution. The color developed by *p*-nitrophenol liberated by phosphatase activity was measured by a Hitachi electrocolorimeter (Model FPW-4) with an S₄₂ filter.

Aldolase—The activity of the enzyme was assayed by the method described by Sibley and Lehninger (5). To 0.25 ml. of substrate solution containing 0.05 *M* fructose-1, 6-diphosphate, 0.25 ml. of 0.56 *M* hydrazine solution (pH 8.6) and 1.0 ml. of 0.1 *M* Tris buffer (pH 8.6) was added. After thermal equilibrium at 36°C, 1.0 ml. of enzyme solution was added and kept at 36°C for 30 minutes. The reaction was stopped by adding 2.0 ml. of 10% trichloroacetic acid. After centrifugation, 1.0 ml. of the supernatant was transferred to a test tube containing 1 ml. of 0.75 *N* NaOH solution. The mixture was kept at room temperature for 10 minutes. After the addition of 1.0 ml. of 0.1% dinitrophenyl hydrazine solution into the test tube, the mixture was incubated at 37°C for 10 minutes. Again 7.0 ml. of 0.75 *N* NaOH was poured into the test tube. The developing brown violet color was measured by a Hitachi electrocolorimeter (Model FPW-4) with an S₅₃ filter.

Alkaline Phosphatase—The procedure is essentially the same as that for acid phosphatase except that the substrate solution contains 0.001 *M* *p*-nitrophenol phos-

phate, 0.01 *M* MgCl₂ and 0.05 *M* Veronal buffer (pH 9).

Arginase—Arginase activity was assayed in a modified Sakaguchi reaction (6) to measure the residual arginine. The reaction medium was composed of 1.0 ml. of 0.001 *M* arginine and 1 ml. of enzyme solution containing 0.0005 *M* MnSO₄ and 0.1 *M* Veronal buffer (pH 9.0). The incubation was carried out at 37°C for 30 minutes, then stopped by the addition of 2.0 ml. of oxine-sulfo mixture (2.5% sulfosalicylic acid, 0.005 *M* glycine and 0.05% oxine). The resulting precipitate was filtered off and 2.0 ml. of the filtrate was kept cold and added to 1 ml. of 2.5% NaOH. After allowing 15 minutes for chilling, 1.0 ml. of hypobromite (1% bromine in 5% NaOH) was mixed in and the total volume was made 10 ml. An orange color developed in several minutes and was measured by means of a Hitachi electrocolorimeter (Model FPW-4) with an S₅₀ filter.

Catalase—For assaying the high activity of catalase in normal and regenerating livers, the following method (7) was adopted: To 20 ml. of cold 0.01 *N* hydrogen peroxide in 0.007 *M* phosphate buffer, 1 ml. of enzyme solution was added. 5 ml. of the aliquots were taken at 0, 3, 6, 9 minutes after the addition of the enzyme, and the aliquots were added to 5 ml. of 2 *N*-H₂SO₄ and titrated with 0.005 *N* potassium permanganate.

In the case of the low activity in tumor tissues, to 5 ml. of the aliquots were added 1 ml. of 5% KI and 0.5 ml. of saturated ammonium molybdate (8). The mixture was allowed to stand for 3 minutes, then titrated with 0.005 *N* sodium thiosulfate. The calculation was carried out using the following equation.

$$K = \frac{1}{t} \log \frac{a}{a-x}$$

K: first order velocity constant

a: initial concentration

x: concentration after t hours

Cathepsin—The activity of cathepsin was estimated by the method of Snoke, Neurath and Lowry (9). In 1 ml. of the substrate solution containing urea denaturated haemoglobin "Takeda" (0.25 g. of haemoglobin and 8.6 g. urea were dissolved in 14 ml. of 0.1 *N* NaOH, incubated at 30°C for 30 minutes, and neutralized by 1 *N* HCl (final pH 3.6)) was mixed 1 ml. of enzyme solution. (The solution was previously diluted with acetate buffer (pH 3.6) to the appropriate concentration. The final concentration of acetate buffer must be *M*/10.) The mixture was then incubated at 37°C for 30 minutes. After the addition of 1.0 ml. of 0.6 *M* trichloroacetic acid, the mixture was kept at 30°C for 30 minutes. The amino acid content in 1 ml. of the supernatant was measured by Folin-

Ciocalteu's method.

Cholin Esterase—The activity was estimated by the technique of Hestrin (10). 1 ml. of the substrate solution containing 0.2 ml. of 0.04 *M* acetyl cholin-HCl and 0.8 ml. of 0.1 *M* phosphate buffer (pH 7.2) and 1 ml. of the enzyme solution were mixed and incubated at 37°C. After 30 minutes, 4 ml. of 1 *M* alkaline hydroxylamine solution was added to the incubation medium and soon after the medium was acidified by the addition of 2.0 ml. of 4 *N* HCl, and the precipitates which occurred were filtered off. 4.0 ml. of the filtrate was mixed with 1 ml. of 0.37 *M* FeCl₃. The brown-violet color developed by the reaction with the residual acetyl cholin was measured by means of a Hitachi electrocolorimeter (Model FPW-4) with an S₅₃ filter.

Glutamic-oxaloacetic Transaminase—The assay method was that described by Caband, Leeper and Wróblewski (11). The total volume of 1.5 ml. medium contained 13.3 mg. DL-aspartic acid, 3 mg. α-ketoglutarate, 10 mg. KH₂PO₄ and 0.5 ml. of enzyme solution. The pH was adjusted to pH 7.4. After incubation at 26°C for 20 minutes, the reaction was stopped by the addition of 2 gtt. of 50% trichloroacetic acid. To the supernatant, 1 gtt. of anilin-citric acid mixture (5 ml. of water, 5 g. of citric acid and 5 ml. of aniline) was added and after 10 minutes, 0.5 ml. of 0.1% di-nitrophenylhydrazine-HCl was also pipetted into the supernatant. The resulting solution was kept at room temperature for 5 minutes, then 2.0 ml. of toluen was mixed with vigorous shaking. 1 ml. of toluen layer was added with 3 ml. of 2.5% KOH-alcohol and the brown color which developed was optically measured by a Hitachi electrocolorimeter (Model FPW-4) with an S₅₀ filter.

Histidase—The activity of this enzyme was measured by the method described by Takeuchi (12) using the determination of the residual histidine. The reaction mixture was composed of 1 ml. of enzyme solution and 1 ml. of substrate solution containing 0.04 *M* histidine and 0.05 *M* phosphate buffer (pH 8.3). After incubation at 37°C for 1 hour, the reaction was stopped by 2.0 ml. of 16% trichloroacetic acid. 1 ml. of the filtrate was mixed with 1 ml. of cupric sulfate mixture (0.075% CuSO₄ 10 ml. + conc. H₂SO₄ 9 ml. + 1 *M* glycine 10 ml. and sufficient water so as to make 100 ml.), 1 ml. of KBrO₃ and 0.5 ml. of saturated bromine water. Then the mixture was incubated at 37°C in the dark. After 1 hour, 3% arsenite solution was added to the mixture until the bromine color was disappeared. Then the mixture was extracted by 5.0 ml. of ethylacetate-xylol mixture (1:1). The aqueous layer and washings were combined and mixed with 2 ml. of 27.2% sodium acetate. The final volume

was made 10 ml. with water. After an incubation at 37°C for 10 minutes, a red color developed. The optical measurement was carried out by a Hitachi electrocolorimeter (Model FPW-4) with an S_{54} filter.

Peptidase—The enzymic activity was determined by a method proposed by one of the authors (Furiya) (13). 1 ml. of the substrate solution containing 0.001 *M* DL-leucyl-m-aminobenzoic acid and 0.05 *M* phosphate buffer (pH 7.4) was mixed with 1 ml. of enzyme solution and incubated at 37°C for 30 minutes. By adding 1 ml. of 10% trichloroacetic acid, the reaction was stopped and the protein fraction was filtered off. To 1 ml. of the cold filtrate 5 *gtt.* of *N*-HCl was added. Two minutes after the addition of 1 *gtt.* of 30% NaNO_2 , 2 ml. of H acid-ammonium sulfite mixture (0.05% H acid, 0.5% NaSO_3 and 2.5% ammonia) was promptly mixed in. The total volume of the mixture was made 10 ml. by adding water. After 10 minutes, the color of the diazotized dye was measured by a Hitachi electrocolorimeter (Model FPW-4) with an S_{53} filter.

Urocanicase—The assay method of this enzyme was that which Edlbacher (14) developed for the determination of the residual urocanic acid. 1 ml. of the enzyme solution and 1 ml. of the substrate solution containing 0.002 *M* urocanic acid and 0.02 *M* phosphate buffer (pH 7.4) were mixed and incubated at 37°C for 1 hour. The reaction was stopped by the addition of 2.0 ml. of 16% trichloroacetic acid. The resulting precipitate was filtered off, and 1 ml. of the filtrate was neutralized by adding 1 ml. of 0.5 *N* NaOH. 1 ml. of this solution was chilled, and then 5.0 ml. of 0.53% Na_2CO_3 and 0.5 ml. of diazoreagent (2 volumes of 0.25% *p*-nitroaniline solution containing 0.03 *N* HCl and 1 volume of 0.5% NaNO_2 were added). The mixture was allowed to stand in the cold for 15 minutes. The resulting orange dye was extracted by 10.0 ml. of butanol. The optical density was measured by a Hitachi electrocolorimeter (Model FPW-4) with an S_{47} filter.

Units of Enzymes and Presentation of Results—To simplify the construction of table, the following symbols will be used to designate the isolated fraction: N=nuclear fraction; M=mitochondrial fraction; P=particulates or microsomal fraction; S=final supernatant.

The results of the enzyme distribution studies will be expressed in units/mg. protein of enzyme containing fraction.

Except for cathepsin and catalase, one unit of enzymic activity refers to the decomposition of 1 μmole of substrate/hour under the conditions of the assay. The molarity of the products of cathepsin action was expressed conventionally in terms of tyrosine equiva-

lents of the color developed with the Folin-Ciocalteu reagent and one unit of catalase refers to the hydrolysis of 1 $\mu\text{mole/min.}$

The distribution patterns of enzymes were expressed by the method originally established by De Duve (1). Fig. 1 is an example of such an expression. Glutamic-oxaloacetic-transaminase of normal rat liver (heavy line) and AH 7974 hepatoma (light line). Ordinate: mean relative specific activity of fractions. The relative specific activity was expressed as the ratio of the specific activity of each fraction per specific activity of the original homogenate.

A unit is according by the specific activity of the homogenate. Abcissa: fractions are represented by their relative nitrogen content measured by the method of Folin-Ciocalteu, in the order in which they are isolated, *i. e.* from left to right: N, M, P, and S.

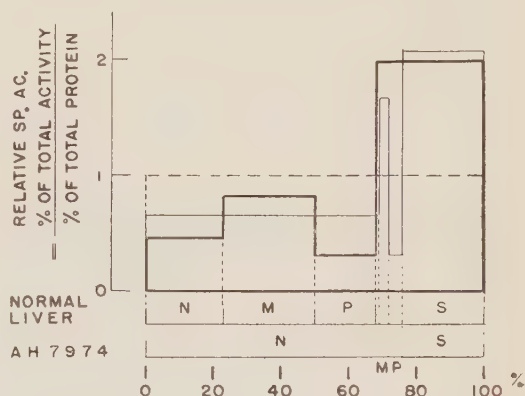


FIG. 1. The distribution patterns of glutamic-oxaloacetic transaminase in normal liver and hepatoma (AH 7974).

The area of each block is thus proportional to the percentage of activity recovered in the corresponding fraction, and its height to the degree of purification achieved over the homogenate.

RESULTS

General Survey of Quantitative Data—Specific activities of the enzymes in homogenates of normal and regenerating livers as well as hepatoma cells are recorded in Fig. 2.

In general, there are two groups of enzymes: those that show very weak activities in cancer homogenates compared with the normal or regenerating livers homogenates, (*e. g.* arginase, catalase, glutamic-oxaloacetic-transaminase, histidase and urocanicase) and those that have equal or sometimes more activ-

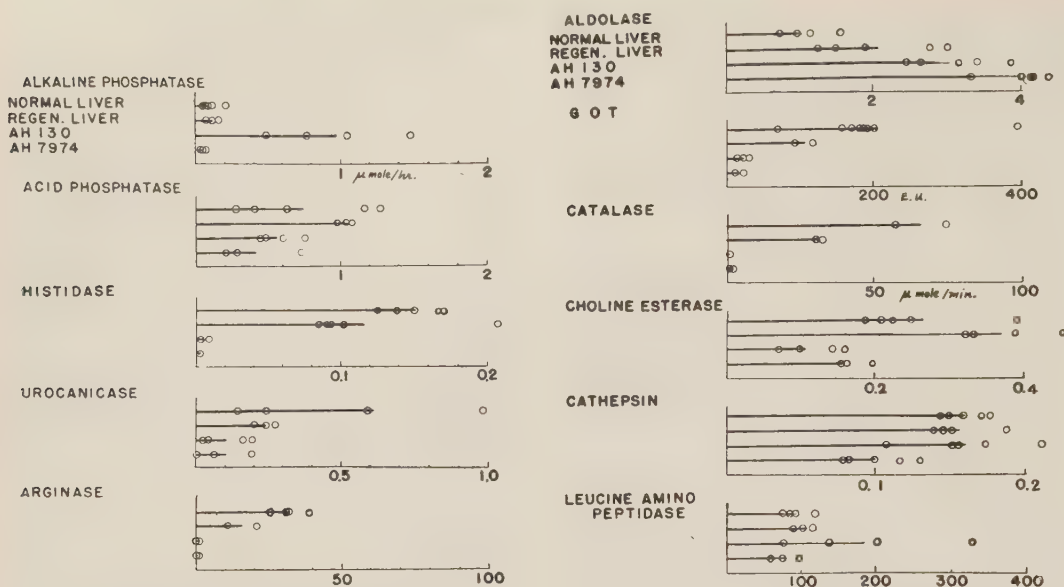


FIG. 2. Specific activities of various enzymes in rat liver homogenates and hepatoma homogenates.

ity in cancer homogenates than in normal or regenerating livers homogenates. (*e. g.* aldolase, cathepsin and leucine amino-peptidase). In most cases, there are no distinct differences of specific activities between the AH 130 strain and the AH 7974 strain of hepatoma. However, in the case of alkaline phosphatase, the specific activities of this enzyme were quite low in AH 7974 homogenates whereas those in AH 130 homogenates were always high in every trial.

From the view point of the distribution patterns of enzymes (Fig. 3), one could divide the enzymes tested into the following three categories: Type 1, the distribution patterns are almost similar in hepatoma cells as well as in normal and regenerating liver cells. (*e. g.* aldolase, choline esterase, glutamic-oxaloacetic-transaminase and urocanicase). Type 2, the distribution patterns are similar in the two types of normal cells and in the two types of tumor cells, but entirely different patterns were observed if normal and tumor cells are compared to each other. (*e. g.* arginase, catalase and peptidase) Type 3, the distribution patterns are similar in normal and regenerating livers but different from strain to strain in tumors. (alkaline phosphatase).

Detailed Analysis of Results Obtained for Each Enzyme

Aldolase—There are several reports concerning the increased activity of this enzyme in cancer cells and in the serum of cancer patients (15, 16). In our data also, normal liver homogenates showed the lowest activities compared with the proliferating tissues. However, the distribution patterns of this enzyme were rather similar in normal and neoplastic tissues, and everytime the supernatant fractions contained the highest activity of this enzyme. This fact might have some relationship to the high activity in the serum of cancer patients since the enzyme may leak from the cell-sap of cancer tissue by the change of permeability of cancer cells.

Acid and Alkaline Phosphatase—The specific activities of acid phosphatase in homogenates of tumor cells were comparatively lower than those in normal cells. The lower activities of the enzyme in particulates fractions are characteristic in neoplastic cells.

As noted before, the specific activities of the AH 130 homogenates were specifically high and the lowest activities were measured in the AH 7974 homogenates. The distribu-

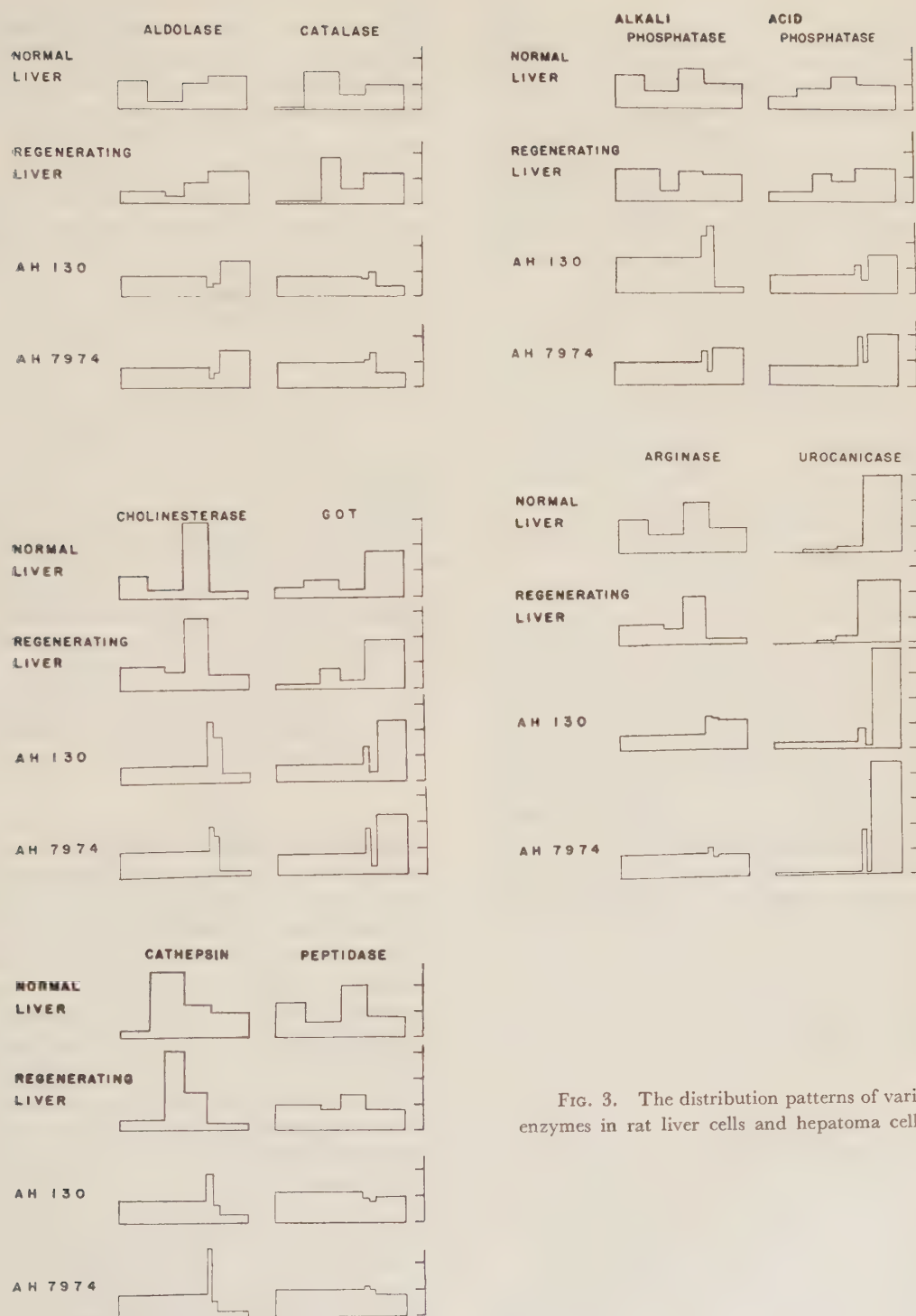


FIG. 3. The distribution patterns of various enzymes in rat liver cells and hepatoma cells.

tion patterns of this enzyme are also characteristic in the AH 130 cells: very low activity was observed in the supernatant fraction whereas the highest activity was found in the microsomal fraction. This pattern is entirely different from that of AH 7974. Katayama already reported the higher activity of this enzyme in the nucleal fraction of AH 130 compared with the same fraction of AH 7974 (17). Although there exists a general idea that different types of malignant tissues demonstrate rather similar protein and enzyme patterns (18), this observation may encourage our working-hypothesis that each strain of tumors has different metabolic patterns since they have a different number of chromosomes.

Arginase—The specific activities of these enzymes in cancer were about one hundredth of the normal tissues. The reason why the lower activity was observed in cancer was discussed by some authors (19).

The difference of the distribution patterns between normal tissues and cancer cells was striking. However, as Schneider, Laird and De Duve have already reported, the use of 0.25 *M* sucrose for the subcellular fractionation resulted the different distribution pattern of arginase (20, 21, 1). Carruthers adopted another fractionation method using glycerol and obtained rather different patterns compared to our results (22).

Catalase—Very low activity of catalase in cancer homogenates may be one of the characteristic features of neoplastic tissues. As the main localization of this enzyme is in the mitochondrial fraction, the weak activity of this enzyme may be attributed either to the decreased number of mitochondria in cancer cells or to the lower content of iron in cancer cells.

Cathepsin—There are no remarkable differences between the specific activities of the hepatoma homogenates and normal liver homogenates. Similarly the distribution patterns of cancer cells were fundamentally the same as those observed in normal livers.

Choline Esterase—There are conflicting reports on the activity of this enzyme in hepatoma: The high activities were observed in

hepatoma *in situ* (23). In our case, comparatively lower activities were observed in ascites hepatoma homogenates. In the distribution pattern, the highest activities in microsomal fractions were characteristic in normal tissues.

Glutamic-oxaloacetic Transaminase—Although there are some reports that the activity of this enzyme in the sera of cancer patients increases according to the development of the tumor, our results show relatively lower activities in hepatoma homogenates. The distribution patterns were similar in normal and neoplastic cells.

Histidase—Like the previous reports by Masayama or by Kishi, the activity of this enzyme was scarcely detected in our experiment (24, 25).

Urocanicase—Voillier observed that an enzyme splitting imidazole ring was reduced in DAB hepatoma (26). As our method to measure urocanicase activity is based on the degree of splitting activity of the imidazole ring, both enzymes are presumably identical ones. This enzyme is located mainly in the supernatant fraction of normal and neoplastic tissues. However, small peaks were observed in cancer mitochondrial fractions.

DISCUSSION

De Duve proposed to divide the mitochondrial fraction into two subfractions. Applying this fractionation method, he proposed four groups of the distribution patterns of various enzymes. The first group comprises cytochrome oxidase, rhodanase and cytochrome reductase. All these enzymes have been found to be associated with the heavier cytoplasmic granules, and may therefore be considered as truly mitochondrial. In the enzyme studied, cathepsin might belong to this group. In the second group, which comprise the microsomal enzymes, are found glucose-6-phosphatase and special β -glucuronidase (opt. pH 5.2–5.3). Arginase and choline esterase in normal tissues seem to belong to this category. The third group of enzymes includes acid phosphatase, ribonuclease and deoxyribonuclease. The peak of specific activ-

ities exists in the light mitochondrial fraction. Cathepsin is included in this group according to De Duve's fractionation. However, in our experiment the light mitochondrial fraction was not isolated because of the paucity of the total mitochondrial fraction in cancer cells. Thus, we could include neither cathepsin nor acid phosphatase in this group. De Duve postulated also that uricase might belong to lysosomes or to yet another class of granules, since uricase is essentially insoluble and might be expected to sediment with particles. The representative enzyme which belongs to the fourth group of the distribution patterns is uricase, whose specific activity is particularly high in lysosomes but relatively high in other particulate fractions.

In our case, urocanicase was especially active in the supernatant fraction. This might be a new group which was not observed in the enzymes studied by De Duve. However, as cited before, the distribution patterns of some enzymes showed several varieties according to the status of the tissue: *e.g.* non-growing, proliferating or malignant. Even in malignant cells, the differences of the strains may change the shape of the distribution patterns like alkaline phosphatase.

SUMMARY

1. The specific activities of a number of enzymes has been investigated in normal and regenerating rat liver homogenates as well as two strains of hepatoma homogenates. Arginase, catalase, glutamic-oxaloacetic-transaminase and histidase showed lower activities in cancer homogenates whereas aldolase was found more active in neoplastic tissue homogenates.

2. The intracellular distribution of the enzymes was compared and divided into three groups: Type 1 has a distribution pattern common to normal and regenerating liver as well as cancer cells (aldolase, choline esterase, glutamic-oxaloacetic-transaminase and urocanicase). Type 2 has a peculiar pattern for cancer cells (arginase catalase and peptidase). Type 3 shows a common pattern for normal tissues but entirely different shapes will be

observed in each strain of hepatoma (*e.g.* alkaline phosphatase).

3. In discussing these results, some conflicting evidence as compared with De Duve's work has also been brought to light.

The work has been supported by grants from the Ministry of Education to Cancer Chemotherapy Research Group. We wish to thank the staff of the Sasaki Institute for their kind supply of AH strains-hepatoma.

REFERENCES

- (1) De Duve, C., *Biochem. J.*, **50**, 172 (1952); **60**, 604 (1955)
- (2) Higgins, G. M., and Anderson, R. M., *Arch. Pathol.*, **12**, 186 (1931)
- (3) Hogeboom, G. H., "Method in Enzymology", Vol. **I**, 16 (1955)
- (4) Ohmori, Y., *Enzymologia*, **4**, 217 (1937); Fujita, H., *J. Biochem.*, **30**, 69 (1939)
- (5) Sibley, J. A., and Lehninger, A. L., *J. Biol. chem.*, **177**, 859 (1949)
- (6) Watanabe, T., *J. Chiba Med. Soc. (Japan)*, **34**, 86 (1958)
- (7) Von Euler, H., and Josephson, K., *Ber. deut. chem. Ges.*, **56**, 1749 (1923)
- (8) Sumner, J. B., and Dounce, A. L., "Method in Enzymology", Vol. **II**, 774 (1955)
- (9) Snoke, J. E., and Neurath, H., *J. Biol. Chem.*, **187**, 127 (1950)
- (10) Hestrin, S., *J. Biol. Chem.*, **180**, 249 (1949)
- (11) Cabau, P., Leeper, R., and Wróblewski, F., *Am. J. Clin. Pathol.*, **26**, 1101 (1956)
- (12) Takeuchi, R., *J. Chiba Med. Soc. (Japan)*, **35**, 2414 (1960)
- (13) Furiya, S., *Symposia on Enzyme Chemistry (Japan)*, **4**, 64 (1950)
- (14) Edlbacher, S., Bauer, H., Stachelin, H. R., and Zeller, A., *Z. physiol. Chem.*, **270**, 158 (1941)
- (15) Sibley, J. A., and Lehninger, A. L., *J. Natl. Cancer Inst.*, **9**, 303 (1949)
- (16) Sibley, J. A., Fleisher, G. A., and Higgins, G. M., *Cancer Research*, **15**, 306 (1955)
- (17) Katayama, H., *Nisshin Igaku (Japan)*, **44**, 649 (1957)
- (18) Angeletti, P. U., Moore, B. W., and Suntzeff, V., *Cancer Research*, **20**, 1592 (1960)
- (19) Rosenthal, O., *J. Biol. Chem.*, **223**, 469 (1956)
- (20) Schneider, W. C., *J. Biol. Chem.*, **185**, 869 (1950)
- (21) Laird, A. K., *Exptl. Cell Research*, **6**, 30 (1952)
- (22) Carruthers, C., *Cancer Research*, **19**, 59 (1959)

- (23) Voillier, G., and Waser, P., *Helv. Physiol. et Pharmacol. Acta*, **8**, C39 (1950) (Japan), **49**, 281 (1958)
- (24) Masayama, T., *Gann (Japan)*, **32**, 303 (1938) (26) Voillier, G., *Helv. Physiol. et Pharmacol. Acta*, **8**, C38 (1950)
- (25) Kishi, S., Tsuruoka, N., and Asano, B., *Gann*

Variation de la Composante Amino-acide des Tissus et Euryhalinité chez *Perinereis cultrifera* Gr. et *Nereis diversicolor* (O. F. Müller)

Par CH. JEUNIAUX, GH. DUCHÂTEAU-BOSSON et MARCEL FLORKIN

(Université de Liège, Biochimie, Prof. M. Florkin, et Station
biologique de Roscoff, Finistère)

(Reçu pour la publication, le 6 avril 1961)

Au cours d'un travail antérieur, nous avons fait remarquer que, chez les Invertébrés marins qui présentent un degré plus ou moins étendu d'euryhalinité, deux mécanismes différents peuvent être responsables de cette faculté d'adaptation (1). Chez des animaux comme la Moule (2) et *Arenicola* (1), le seul mécanisme mis en jeu est la variation de concentration des constituants intracellulaires, notamment de la composante amino-acide. Chez d'autres Invertébrés euryhalins, ce mécanisme est complété par une osmorégulation, c'est-à-dire par un mécanisme d'ionorégulation maintenant la concentration osmotique du milieu intérieur à une valeur supérieure à celle du milieu extérieur, quand ce dernier est moins concentré que l'eau de mer (*Carcinus* (3, 4), *Eriocheir* (5)).

Ce qui précède fait supposer que le mécanisme intracellulaire représente le mécanisme le plus ancien rendant compte d'un degré plus ou moins marqué d'euryhalinité, le mécanisme osmorégulateur étendant encore vers les régions de basses salinités du milieu aquatique, les capacités euryhalines des Invertébrés marins qui l'auraient secondairement acquis.

Le cas des espèces marines plus ou moins euryhalines que présentent les genres *Nereis* et *Perinereis* offre dans ce domaine un sujet d'études particulièrement adéquat. *Nereis diversicolor* (O. F. Müller) est le seul Invertébré marin connu, en dehors des Crustacés, comme présentant une osmorégulation lorsqu'il vit dans l'eau saumâtre où il supporte une dilution poussée jusqu'à celle de l'eau de mer diluée cinq fois (6). *Perinereis cultrifera* Gr.,

bien qu'étant poecilomotique (7), présente cependant un degré non négligeable d'euryhalinité qui l'oppose à *Nereis pelagica* L., elle aussi poecilomotique (6), mais dont l'aire de distribution est limitée à l'eau de mer, tandis qu'on trouve *Perinereis cultrifera* dans certains estuaires (8).

Nous avons donc mesuré chez *N. diversicolor* et *P. cultrifera* les variations de pression osmotique des milieux extracellulaires et celles de la concentration en quinze acides aminés libres intracellulaires, au cours de l'adaptation à des milieux hypotoniques.

METHODES

Les animaux utilisés pour ces expériences ont été choisis intacts, et ceux dont la partie postérieure du corps avait été abandonnée par autotomie en cours d'expérience ont été éliminés. D'autre part, afin d'éviter de trop grandes perturbations, les vers en expérience ont été conservés dans des aquariums garnis d'une couche de 3 à 4 cm de sable ou de vase provenant de l'endroit de récolte de ces vers. Toutes les expériences ont été réalisées au mois de juin 1960.

Les individus appartenant à l'espèce *Perinereis cultrifera* ont été recueillis dans le sable de la grève de Roscoff, en face du laboratoire maritime. Un lot témoin a été conservé en eau de mer courante, pendant que les vers du second lot étaient transférés dans des aquariums recevant une eau de mer de plus en plus diluée, jusqu'à atteindre la proportion d'un volume d'eau de mer pour un volume d'eau douce. Cette période d'adaptation a duré 6 heures. Les vers ont ensuite été conservés pendant 3 jours dans un mélange à parties égales d'eau de mer et d'eau douce, bien aéré et constamment renouvelé.

Vers témoins et vers adaptés à l'eau de mer diluée ont été utilisés pour le prélèvement des liquides organiques extracellulaires et pour la préparation des

broyats de tubes musculo-cutanés. Six individus ont fourni environ 1.5 ml. de mélange de sang et de liquide coelomique, dont la pression osmotique a été évaluée par mesure de l'abaissement cryoscopique. Pour la préparation des tubes musculo-cutanés, chaque animal, essoré sur papier filtre, a été ouvert, au moyen de ciseaux fins, sur toute sa longueur le long du vaisseau dorsal. Après élimination du 1/3 postérieur du corps, pauvre en muscles, de la tête et du pharynx, le contenu du tube musculo-cutané (tube digestif, néphridies) a été enlevé à la pince et par grattage au scalpel. La préparation a été ensuite essorée, lavée dans de l'eau de mer isotonique glacée, soigneusement essorée de nouveau, pesée sans délai, et plongée instantanément dans un volume connu d'eau distillée bouillante, afin d'inactiver les enzymes. Nous avons veillé à ce que l'ensemble de ces manipulations n'ex-cède jamais 10 minutes.

Les individus appartenant à l'espèce *Nereis diversicolor* ont été récoltés dans la vase de l'Aber de Roscoff. Pendant qu'une partie des animaux (lot témoin) était gardée dans de l'eau de mer courante, l'autre partie

a été adaptée progressivement à de l'eau de mer de plus en plus diluée, de telle sorte que, après 36 heures environ, la proportion d'eau de mer était de 1/5. Les vers ont été maintenus pendant 4 jours dans de l'eau de mer diluée 5 fois, bien aérée et constamment renouvelée.

La récolte du mélange de sang et de liquide coelomique a été réalisée par prélèvement au moyen d'une pipette capillaire. La préparation des tubes musculo-cutanés a été réalisée comme pour l'espèce précédente.

Les différentes préparations musculo-cutanées constituant un même lot ont été rassemblées, avec l'eau où on les a ébouillantées, et homogénéisées au moyen d'un Waring-blendor. La purée obtenue a été dialysée pendant 24 heures à 0°C contre un volume 10 fois supérieur d'eau distillée. Le dialysat, évaporé à sec, a été hydrolysé par chauffage à reflux pendant 24 heures en présence d'acide chlorhydrique 6*N*. Les résultats sont réunis dans les tableaux I et II, tandis que les tableaux III et IV rassemblent les différentes données numériques.

TABLEAU I
*Variation de concentration de 15 acides aminés libres intracellulaires,
au cours de l'adaptation à des milieux dilués
(mg. d'acide aminé p. 100 g. de tissu frais)*

	<i>Perinereis cultrifera</i>			<i>Nereis diversicolor</i>	
	Eau de mer		Eau de mer diluée 2 fois	Eau de mer	Eau de mer diluée 5 fois
	1	2			
Alanine	305.7	209.6	104.2	323.2	27.6
Arginine	2.8	3.1	1.3	2.1	2.1
Ac. aspartique	96.4	80.2	87.3	94.9	41.4
Ac. glutamique	217.3	168.9	115.9	210.5	91.3
Glycocolle	1329.6	1416.7	727.7	433.6	50.1
Histidine	24.6	22.6	9.5	31.3	11.0
Isoleucine	11.8	9.2	4.9	24.8	4.9
Leucine	15.4	12.2	6.6	22.8	6.0
Lysine	11.1	8.6	5.5	37.0	29.6
Méthionine	30.8	31.0	11.0	40.2	8.5
Phénylalanine	6.8	6.5	4.6	8.6	2.8
Proline	290.7	245.2	203.0	527.2	57.7
Thréonine	94.3	88.9	42.8	51.4	16.5
Tyrosine	10.5	9.9	6.9	24.7	5.5
Valine	24.4	22.4	12.8	44.9	6.5
Total	2472.2	2335.0	1344.0	1877.2	361.5

TABLEAU II

Variation de concentration de 15 acides aminés libres intracellulaires,
au cours de l'adaptation à des milieux dilués

(millimoles d'acide aminé p. 100 g. de tissu frais)

	<i>Perinereis cultrifera</i>			<i>Nereis diversicolor</i>	
	Eau de mer		Eau de mer diluée 2 fois	Eau de mer	Eau de mer diluée 5 fois
	1	2			
Alanine	3.43	2.35	1.17	3.63	.31
Arginine	.02	.02	.01	.01	.01
Ac. aspartique	.72	.60	.66	.71	.31
Ac. glutamique	1.48	1.15	.79	1.43	.62
Glycocolle	17.71	18.87	9.69	5.78	.67
Histidine	.16	.15	.06	.20	.07
Isoleucine	.09	.07	.04	.19	.04
Leucine	.12	.09	.05	.17	.05
Lysine	.08	.06	.04	.28	.20
Méthionine	.21	.21	.07	.27	.06
Phénylalanine	.04	.04	.03	.05	.02
Proline	2.53	2.13	1.76	4.58	.50
Thréonine	.79	.75	.36	.43	.14
Tyrosine	.06	.05	.04	.14	.03
Valine	.20	.19	.11	.38	.06
Total	27.64	26.73	14.88	18.25	3.09
	Moy : 27.18 Différence=0.12 mole/litre (soit environ 0.2°C)			Différence=0.15 mole/litre (soit environ 0.3°C)	

TABLEAU III

Perinereis cultrifera

Abaissement	cryoscopique (Δ)	Nombre d'individus	Poids de tissu frais g.	Eau g. p. 100 g. de tissu frais	mg. acides aminés p. 100 g. de tissu frais	mg acides aminés p. 100 g. d'eau
	Eau de mer					
Δe	-2.08°C	10	10.3281	76.5	2472.2	3175
Δi	-2.06°C	8	10.4974	74.9	2335.0	
	Eau de mer diluée 2 fois			moy. 75.7	moy. 2403.6	
Δe	-1.12°C					
Δi	-1.17°C	8	12.0324	82.8	1344.0	1623

Variation de concentration des acides aminés libres intracellulaires :

a) mg. pour 100 g. d'eau : 3175-1623=1552 mg./100 g. d'eau

b) résultant uniquement de la variation de teneur en eau : 3175-2903=272 mg./100 g. d'eau

c) non expliquée par la variation de teneur en eau : 1552-272=1280 mg./100 g. d'eau

TABLEAU IV
Nereis diversicolor

Abaissement	cryoscopique (Δ)	Nombre d'individus	Poids de tissu frais g.	Eau g. p. 100 g. de tissu frais	mg. acides aminés p. 100 g. de tissu frais	mg. acides aminés p. 100 g. d'eau
Δe	Eau de mer -2.08°C	44	7.4424	75.8	1877.2	2477
Δe	Eau de mer diluée 5 fois -0.43°C	36	8.7739	82.4	361.5	439
Δi	-1.04°C					

Variation de concentration des acides aminés libres intracellulaires :

- a) mg. p. 100 g d'eau : 2477-439=2038
- b) résultant uniquement de la variation de teneur en eau : 2477-2278=199
- c) non expliquée par la variation de teneur en eau : 2038-199=1839 (90 p. 100)

DISCUSSION

Le tableau III montre que, dans l'eau de mer diluée deux fois, représentant la limite extrême de la tolérance à la dilution chez *Perinereis cultrifera*, la modification d'hydratation des muscles de cette espèce est faible, bien que la dilution du sang corresponde à une variation d'abaissement cryoscopique de 1°C environ, c'est-à-dire presque exactement à la valeur de la variation du milieu extérieur. La variation de concentration de la composante amino-acide (dont 82 p. 100 ne sont pas expliqués par la modification d'hydratation) correspond à une variation d'abaissement cryoscopique de 0.2°C environ.

Dans le cas de *Nereis diversicolor* (tableau IV), la forte dilution du milieu extérieur (de -2.08° à -0.43°C, soit une variation de 1.65°C) est compensée par l'osmorégulation du milieu intérieur à concurrence de 0.61°C (la valeur de l'abaissement cyoscopique du sang étant de -1.43°C), et par la diminution de concentration des acides aminés intracellulaires à concurrence de 0.3°C environ.

Les cas de *Perinereis cultrifera* et de *Nereis diversicolor* se rangent donc dans les deux catégories de mécanisme assurant l'euryhalinité : le premier se situant comme ceux de *Mytilus* et d'*Arenicola* parmi les organismes marins dont l'euryhalinité dépend uniquement d'une régulation intracellulaire à laquelle la modification de la composante amino-acide contri-

bue pour une part importante, le second se situant, comme *Carcinus* et *Eriocheir*, parmi les organismes dont l'euryhalinité, plus étendue que celle de la première catégorie, relève à la fois d'une régulation intracellulaire et d'une osmorégulation du milieu intérieur.

Beadle (10), considérant la théorie de Schlieper (6) selon laquelle les espèces homéosmotiques présentent une consommation d'oxygène plus élevée dans les milieux dilués, contrairement aux poecilosmotiques, observe au cours de l'adaptation à l'eau de mer diluée une augmentation de consommation d'oxygène aussi bien chez le poecilosmotique *Perinereis cultrifera* que chez l'homéosmotique *Nereis diversicolor*, l'augmentation de consommation d'oxygène étant plus élevée chez *Nereis diversicolor*. Il conclut que *Perinereis cultrifera* et *Nereis diversicolor* doivent leur euryhalinité au même mécanisme, plus développé chez le second, mécanisme qu'il tient pour "a fundamental property of living cells, which has become more highly developed in certain forms". On peut proposer une interprétation de l'augmentation de consommation d'oxygène chez les animaux euryhalins mentionnés ci-dessus, qu'ils soient poecilosmotiques ou homéosmotiques, par l'existence du mécanisme commun de l'abaissement, dans les milieux dilués, de la composante amino-acide intracellulaire qui limite la pénétration d'eau dans les cellules.

L'abaissement de la composante amino-

acide intracellulaire est vraisemblablement le résultat d'une métabolisation d'acides aminés, avec augmentation de consommation d'oxygène. Le transport actif mis en jeu dans l'osmorégulation du milieu intérieur peut évidemment rendre compte d'une autre portion d'oxygène consommé, comme c'est le cas chez *Nereis diversicolor*, qui cumule la régulation intracellulaire et l'osmorégulation du milieu intérieur.

RÉSUMÉ

Deux espèces d'Annélides marines ont été étudiées : *Perinereis cultrifera*, peu euryhaline, et *Nereis diversicolor*, très euryhaline. Cette différence de degré d'euryhalinité résulte de l'existence, chez *Nereis diversicolor*, d'une osmorégulation du milieu intérieur, dont les effets concourent à compenser la différence de pression osmotique entre cellules et milieu extérieur. Ce mécanisme s'ajoute à un autre mécanisme présent chez les deux espèces, qui met en jeu l'abaissement de la concentration intracellulaire. Chez les deux espèces étudiées, la diminution de concentration de la composante amino-acide intracellulaire contribue pour une part importante à cet abaissement de concentration.

SUMMARY

Among the two species of marine Annelids

studied, *Perinereis cultrifera* and *Nereis diversicolor*, the latter is more euryhaline than the former. This is the result of the existence, in *Nereis diversicolor*, of an osmoregulation of the internal medium, which concurs towards the compensation of the osmotic difference between the cells and the external medium. This mechanism adds its effects to the lowering of the intracellular concentration, a mechanism common to both species and in which the change of concentration of free intracellular aminoacids plays an important part.

BIBLIOGRAPHIE

- (1) Duchâteau, Gh., Jeuniaux, Ch., et Florkin, M., *Arch. Internat. Physiol. Bioch.*, **69**, 30 (1961)
- (2) Potts, W. T. W., *J. exptl. Biol.*, **35**, 749 (1958)
- (3) Duchâteau, Gh., et Florkin, M., *J. de Physiol.*, **48**, 520 (1956)
- (4) Shaw, J., *J. exptl. Biol.*, **35**, 920 (1958)
- (5) Duchâteau, Gh., et Florkin, M., *Arch. Internat. Physiol. Bioch.*, **63**, 249 (1955)
- (6) Schlieper, C., *Z. vergl. Physiol.*, **9**, 478 (1929)
- (7) Wells, G. P., et Ledingham, I., *J. exptl. Biol.*, **17**, 337 (1940)
- (8) Hesse, R., Allee, W. C., et Schmidt, K. P., "Ecological animal geography," New York, Wiley (1937)
- (9) Duchâteau, Gh., et Florkin, M., *Arch. Internat. Physiol. Bioch.*, **62**, 487 (1954)
- (10) Beadle, L. C., *J. exptl. Biol.*, **8**, 211 (1931)

Studies on Bromelain*

II. Its Activation and Fractionation

By SHOSHI OTA, TSEN-HUI FU and RYOZO HIROHATA

(From the Laboratory of Protein Chemistry, Yamaguchi Medical School, Ube)

(Received for publication, February 10, 1961)

Bromelain, a proteolytic enzyme found in the juice of the fruit, leaves and stems of the pineapple, *Ananas comosus* (L.) Merr., is long known under the name of "bromelin". Its behavior toward activation, inhibition or the synthetic substrates has been considered to be similar to that of papain (1-5).

Bergmann and his coworkers (3) found, however, phenylhydrazine to inhibit the hydrolytic activity of bromelain toward certain synthetic substrates but not gelatin. They claim there exist two types of protease in bromelain. No satisfactory information, however, has yet been given on whether or not the clotting of milk and the hydrolysis of protein and peptide are brought about by one and the same enzyme. Masuda (6) demonstrated that papain activated by cyanide was apparently inhibited by either DFP** or aldehyde reagents, while that by cysteine, thioglycolate or hydrogen sulfide was hardly affected. Concerning to such properties of bromelain no description has so far been given. Heinicke and Mori (7) have demonstrated the inhibition of bromelain by DFP. Recently Murachi and Neurath (8) investigated its activity, chromatographic

fractionation and action on glucagon and oxidized insulin. Fu (9) has also made a report on Formosan bromelain.

The purpose of the present investigation was to obtain more detailed information about the milk clotting, the hydrolysis of protein and synthetic substrates, and the optimal condition for the synthesis of hippuryl anilide by bromelain. The electrophoresis and column chromatography of the enzyme prepared from the *Ananas* cultivated in Amamioshima and in Formosa have also been studied.

EXPERIMENTAL

Materials and Methods—For the isolation of the enzyme, Greenberg and Winnick's procedure (4) was slightly modified as follows: The pineapple juice from Amamioshima*** or Formosa was filtered with the aid of the super-cel, and adjusted to pH 6 with 10% NaOH. The 10% NaCN solution was added to the concentration of 0.02 M and the solution was made 40 per cent saturated with respect to $(\text{NH}_4)_2\text{SO}_4$. The resulting precipitate was centrifuged and washed with 40 per cent saturated $(\text{NH}_4)_2\text{SO}_4$. The dissolving and precipitation was repeated once more and finally the precipitate was redissolved in 0.02 M NaCN and precipitated by the addition of 3 volumes of acetone. It was centrifuged, collected on a Buchner funnel, washed with acetone, followed by ether, and dried in a vacuum desiccator. The bromelain used for action on synthetic substrates, electrophoresis and column chromatography was further dialyzed against *aq. dest.* overnight at a low temperature.

Gelatin, skim-milk, BAL, PCMB and DFP were prepared from commercial products.

*** The fresh pineapples were mailed by air from Amamioshima, for which we are deeply indebted to Drs. M. Tsukasa and T. Yasutsu.

*This work was presented at the Chugoku and Shikoku Local Meeting of Biochemical Society, Hiroshima, May 1960 and at the 33rd Annual Meeting of the Biochemical Society, Tokyo, November 1960.

**The abbreviations used are: BAL, 2,3-dimercaptopropanol; PCMB, *p*-chloromercuribenzoate; DFP, diisopropylfluorophosphate; BAA, benzoyl-L-arginine amide; BAOMe, benzoyl-L-arginine methyl ester; Bz-DL-Hmarg Am, benzoyl-DL-homoarginine amide; Bz-L-Agb Am, benzoyl-L- α -amino- γ -guanidinobutyryl amide; EDTA, ethylenediamine tetraacetate.

BAA was a product from Sigma Chemical Company. Bz-DL-Arg Am, Bz-DL-Hmarg Am, Bz-L-Lys Am, Bz-L-Orn Am, Bz-L-Agb Am and Bz-L-Agb ethyl ester were kindly supplied by Miss K. Kitagawa, Laboratory of Biochemistry, Faculty of Science, Kyushu University, to whom our sincere thanks are due. Other synthetic substrates were prepared in our laboratory.

Cellulose powder (300 mesh) was purchased from Toyo Roshi Co. and treated with 0.1M anhydrous ethanolic hydrochloric acid by the method of Flodin and Kupke (10). CM-cellulose and DEAE-cellulose were prepared from the Toyo Roshi Cellulose powder according to the method described by Peterson and Sober (11). Hydroxylapatite was prepared after Tiselius *et al.* (12).

Measurement of Enzymatic Activity—Milk clotting was measured by the ordinary method and the activity was indicated by $1/t$, where t is the time in minutes to produce clotting. Gelatin hydrolysis was measured by the alkali titration method of Grassmann and Heyde (13). As to the determination of the peptidase activity of bromelain, the hydrolysis rates were followed according to Conway's micro-diffusion analysis method (14) or Rosen's ninhydrin method (15) for BAA and other amide substrates, and Hestrin's method (16) for BAOMe and other esters. Proteolytic coefficients were estimated from $C_1 = k_1/e$ for amide substrates and $C_0 = k_0/e$ for ester substrates, where k_1 and k_0 denote the reaction constant of the first order and zero order respectively, i. e., $k_1 = (1/\text{min.}) \ln (100/100 - \text{per cent hydrolysis})$, $k_0 = (\text{rate of hydrolysis})/\text{min.}$, and e is protein concentration in mg. of N per ml.

The rate of synthesis of hippuryl anilide was estimated by the determination of the disappearance of aniline by Korenman and Ganichef (17).

The column (1.2×30 cm.), packed with ethanolic-acid treated cellulose powder in phosphate buffer (pH 6.8, $\mu = 0.1$) was used. The electrophoresis was carried out for 5 hours at 600 volt. current in the same buffer at 4°C.

Column chromatography was carried out essentially as described by Hirs *et al.* for IRC 50 (18), by Ellis and Simpson for cellulose (19) and by Tiselius *et al.* for hydroxylapatite (12). Proteins were estimated by Rosen's ninhydrin method (15).

RESULTS AND DISCUSSION

Effect of Cysteine, Cyanide, BAL and EDTA

—When cysteine, cyanide or BAL was added to the reaction mixture, the enzyme was much activated as shown in Fig. 1 and a full

activation of the enzyme was obtained with cysteine and EDTA.

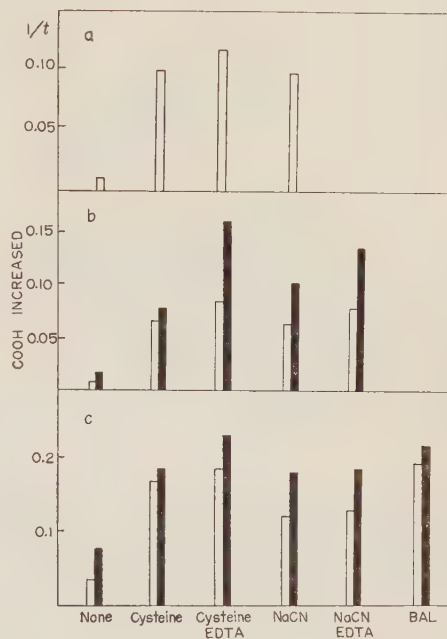


FIG. 1 Effect of cysteine, cyanide, BAL and EDTA. (cysteine 0.005 M, NaCN 0.01 M, BAL 0.01 M, EDTA 0.001 M)

a) Milk clotting: enzyme concentration 0.1 per cent, temperature 30°C.

b) Hydrolysis of gelatin: substrate concentration 2 per cent, enzyme concentration 0.05 per cent, 1/20 M citrate buffer (pH 5.3), temperature 38°C. empty columns 4 hours incubation, full columns 20 hours incubation.

c) Hydrolysis of BAA and BAOMe: enzyme concentration 0.034 mg. N/ml., temperature 38°C, empty columns, BAA 0.01 M, pH 5.3, 1/20 citrate buffers full columns, BAOMe 0.01 M, pH 7.0, 1/20 phosphate buffer.

Effect of pH—The pH activity curves for the hydrolysis of gelatin, mugiline β , hippuryl amide, BAA and BAOMe were presented in Figs. 2 and 3. The pH for the optimum activity was about 5 for gelatin and hippuryl amide, pH 5-7 for BAA and pH 7 for mugiline β and BAOMe.

Effect of Temperature—As shown in Figs. 4 and 5, the highest activity was obtained when incubated at 50°C.

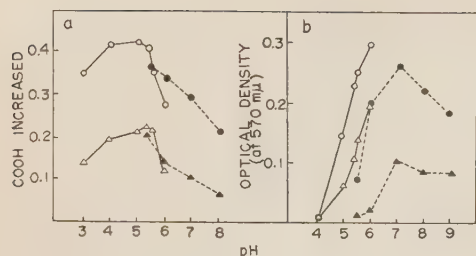


FIG. 2. Effect of pH.

○, △ *M*/20 citrate buffer, ●, ▲ *M*/20 phosphate buffer, △, ▲ 4 hours incubation, ○, ●, 20 hours incubation
 a) Gelatin: substrate concentration 2 per cent, enzyme concentration 0.05 per cent, cysteine 0.025 *M*, EDTA 0.001 *M*, temperature 38°C.
 b) Mugiline β: substrate concentration 0.8 per cent, enzyme concentration 0.02 per cent, NaCN 0.01 *M*, temperature 38°C.

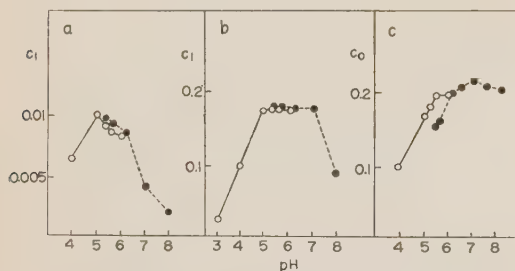


FIG. 3. Effect of pH.

○, *M*/20 citrate buffer, ●, *M*/20 phosphate buffer, enzyme concentration 0.034 mg. *N*/ml, temperature 38°C.

- a) Hippuryl amide 0.025 *M*, BAL 0.01 *M*.
 b) BAA 0.01 *M*, BAL 0.01 *M*.
 c) BAOMe 0.01 *M*, cysteine 0.005 *M*, EDTA 0.001 *M*.

Effect of PCMB, DFP, Hydroxylamine and Phenylhydrazine—As shown in Fig. 6 the cyanide-activated bromelain was inhibited by PCMB, DFP, hydroxylamine or phenylhydrazine, although it was not so remarkable on the milk clotting, while cysteine-activated one was hardly inhibited by these reagents.

Synthesis of Hippuryl Anilide—The experiment for synthesis of hippuryl anilide was carried out substantially as described by Doherty and Popenoe (20). As shown in Fig. 7 the optimal condition for synthesis of hippuryl anilide was in pH 4.4 acetate buffer containing 0.005 *M* cysteine and 0.001 *M* EDTA

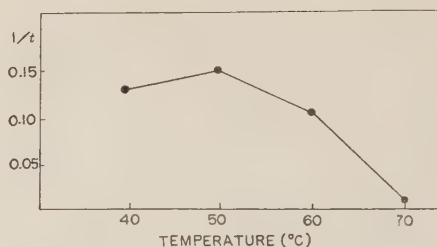


FIG. 4. Effect of temperature (milk clotting).

Enzyme was incubated with cysteine and EDTA at each temperature for ten minutes, and then incubated with milk (adjusted to pH 5.3) at 30°C. Final concentration were 0.1 per cent for enzyme, 0.005 *M* for cysteine and 0.001 *M* for EDTA.

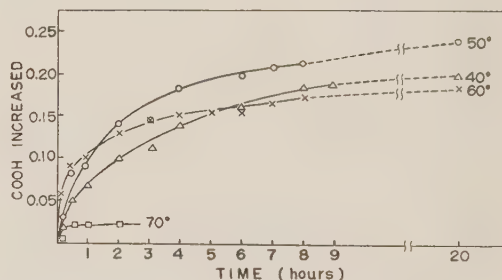


FIG. 5. Effect of temperature (gelatin hydrolysis).

Substrate concentration 2 per cent, enzyme concentration 0.05 per cent. The mixture was incubated in *M*/20 citrate buffer (pH 5.3), contained cysteine 0.0025 *M* and EDTA 0.001 *M*. Temperature 38°C.

Activation of Bromelain on Various Synthetic Substrates—In Table I are summarized C_1 or C_0 and relative rates of hydrolysis of various synthetic substrates. BAA and BAOMe are the best substrates of papain, which were also most intensively hydrolyzed by bromelain. The hydrolysis took place at 0.01 *M* initial concentration of substrates in decreasing order as follows: BAA > DL-BAA > DL-Hmarg Am > Bz-L-Orn Am > Bz-L-Lys Am > Bz-L-AgB Am. The hydrolysis of hippuryl amide was not so intensive, yet significant enough, and this result, however, was at variance with that reported by Bergmann and Fruton

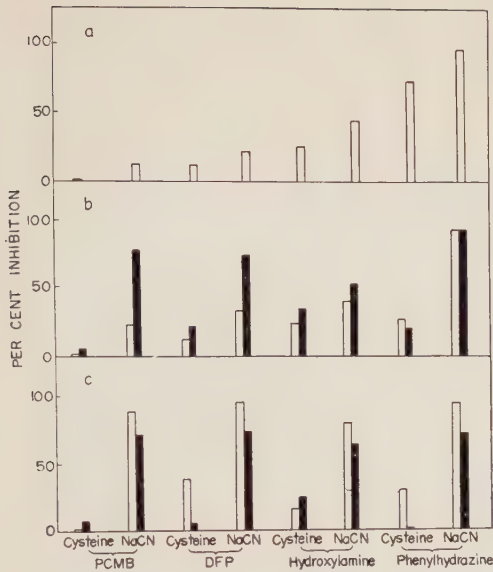


FIG. 6. Effect of PCMB, DFP, hydroxylamine and phenylhydrazine.

(cysteine 0.005 *M*, NaCN 0.01 *M*, PCMB 0.0001 *M*, DFP 0.01 *M*, hydroxylamine 0.005 *M*, phenylhydrazine 0.005 *M*)

a) Milk clotting: enzyme concentration 0.1 per cent, temperature 30°C.

b) Hydrolysis of gelatin: substrate concentration 2 per cent, enzyme concentration 0.05 per cent, 1/20 *M* citrate buffer (pH 5.3), temperature 38°C, empty columns, 4 hours incubation: full columns, 20 hours incubation.

c) Hydrolysis of BAA and BAOMe: enzyme concentration 0.034 mg. N/ml., temperature 38°C. empty columns, BAA 0.01 *M*, pH 5.3, 1/20 citrate buffer, full columns, BAOMe 0.01 *M* pH 7.0, 1/20 phosphate buffer.

(3) who indicated that hippuryl amide had not been hydrolyzed by the enzyme. As shown also in Table I the relative rate of hydrolysis by bromelain is comparable with that of papain. In general, both enzymes have a similar specificity toward the synthetic substrates.

Electrophoresis and Column Chromatography—The paper electrophoregrams of bromelain are shown in Fig. 8. Only one band appeared in Amamioshima bromelain, while two bands in Formosan one, although traces of the insoluble substances remained at the starting line. The activities of both bands in For-

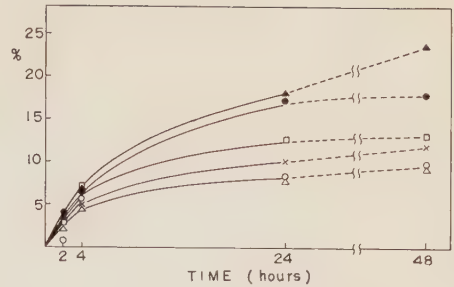


FIG. 7. Synthesis of hippuryl anilide.

Substrate concentration 0.4 *M*, enzyme concentration 0.8 per cent, temperature 38°C.

△, no activator, pH 5.3, ○, 0.01 *M* NaCN, pH 5.3, ×, 0.01 *M* NaCN, 0.001 *M* EDTA, pH 5.3, □, 0.005 *M* cysteine, pH 5.3, ●, 0.005 *M* cysteine, 0.001 *M* EDTA, pH 5.3, ▲, 0.005 *M* cysteine, 0.001 *M* EDTA, pH 4.4.

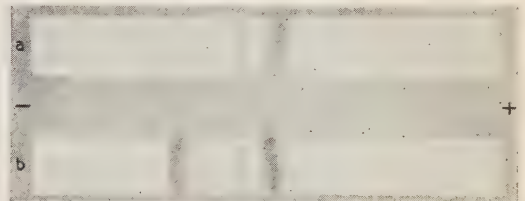


FIG. 8. Paper electrophoregram of bromelain from Amamioshima (a) and from Formosa (b).

(Veronal buffer, pH 8.6, $\mu=0.1$, 15 volt./cm. at 4°C for 6 hours)

mosan bromelain were measured on the respective eluate and found that the activity of the band moved further to anode is 2 or 3 times higher than that in the another band.

Fig. 9 shows the elution diagrams of the zone electrophoresis carried out with dialyzed bromelain. One ml. buffer solution containing 5 mg. enzyme was applied. Three peaks were found as shown in the diagram and the activity of the milk clotting and BAA-hydrolysis was recognized only in the second peak.

Column chromatography was carried out with IRC 50 and presented in Fig. 10. The enzyme activity was found in the non-adsorbed fraction of both Formosan and Amamioshima bromelain. In the eluate with 0.2 *M* buffer no activity was recognized. The same pattern was also obtained with CM-cellulose. As shown in Figs. 11 and 12, the column

Action of Bromelain on Synthetic Substrates

Substrate	C ₁	Relative rate of hydrolysis by bromelain	Relative rate ¹⁾ of hydrolysis by papain
BAA	0.180	100	100
D,L-BAA	0.140	81.1	57
Bz-D,L-Hmarg Am	0.092	51.1	107
Bz-L-Agb Am	0.037	20.6	22
Bz-L-Orn Am	0.090	50.0	76
Bz-L-Lys Am	0.081	45.0	79
Bz-L-His Am	0.009	5.0	9.2 (Cbz-)
Bz-L-Phe Am	0.000	0	—
Hippuryl Am	0.011	6.1	1.2
L-Leu Am	0.001	0.6	3.3

C ₀			
BAOMe	0.216	100	100
Bz-L-Agb OEt	0.039	18.1	10
Bz-L-His OEt	0.077	35.6	—

Substrate concentration, 0.01 *M* with respect to the L-isomer: enzyme concentration 0.0324 mg. N/ml.: cysteine 0.005 *M*, EDTA 0.001 *M*. The mixture was incubated in pH 5.3, *M*/20 citrate buffer for amide substrates or in pH 7, *M*/20 phosphate buffer for ester substrates, at 38°C.

1) Calculated from the results of Smith (23) and Izumiya *et al.* (21, 22).

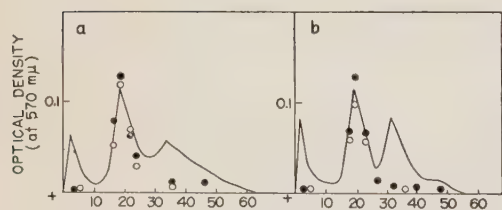


FIG. 9. Zone electrophoretic patterns of bromelain from Amamioshima (a) and from Formosa (b). (cellulose column 1.2×30 cm., phosphate buffer, $\mu=0.1$, pH 6.8, 600 volt. 5 hours)
●, milk clotting ○, BAA hydrolysis
The activities are shown on arbitrary scales.

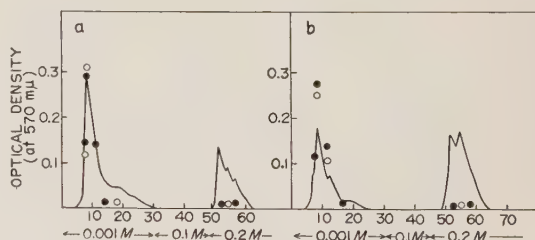


FIG. 10. Column chromatograms of bromelain from Amamioshima (a) and from Formosa (b) with IRC 50. (0.9×30 cm., phosphate buffer, pH 6.8)
●, milk clotting ○, BAA hydrolysis
The activities are shown on arbitrary scales.

chromatograms with hydroxylapatite and DEAE-cellulose showed activity in the fraction eluted with 0.1 or 0.2 *M* phosphate buffer, however no activity in the non-adsorbed fraction, which showed a strong ninhydrin reaction, especially when the hydroxylapatite

was used. Possibly the enzyme was partly destroyed. In column chromatography, there was no great difference between Amamioshima and Formosan bromelain such as was seen in paper chromatography.

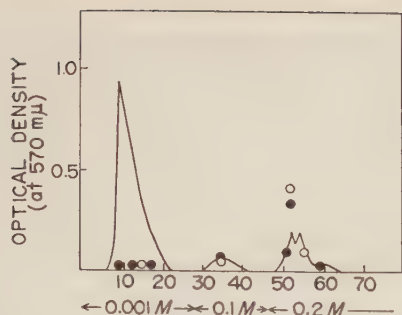


FIG. 11. Column chromatogram of bromelain from Formosa with hydroxylapatite.

(0.9×15 cm., phosphate buffer, pH 6.8)

●, milk clotting ○, BAA hydrolysis

The activities are shown on arbitrary scales.

SUMMARY

1. Milk clotting and the hydrolysis of protein and synthetic substrates by means of bromelain were tested.

2. These actions were stimulated by cysteine, cyanide or by BAL. The full activation was obtained with cysteine and EDTA at 50°C .

3. Bromelain activated by cyanide was inhibited by PCMB, DFP, hydroxylamine or phenylhydrazine, while that by cysteine was hardly inhibited by the above reagents.

4. The optimal condition for the synthesis of hippuryl anilide was determined.

5. Among various synthetic substrates tested benzoyl-L-arginine amide and benzoyl-L-arginine methyl ester were found to be most rapidly hydrolyzed.

6. The electrophoresis and column chromatography of bromelain from Amamioshima and from Formosa were studied.

REFERENCES

- (1) Willstätter, R., Grassmann, W., and Ambros, O., *Z. physiol. Chem.*, **151**, 286, 307 (1926)
- (2) Maschmann, E., *Z. physiol. Chem.*, **228**, 141 (1934)
- (3) Bergmann, M., Fruton, J. S., and Fraenkel-Conrat, H., *J. Biol. Chem.*, **119**, 35 (1937)
- (4) Greenberg, D. M., and Winnick, T., *J. Biol. Chem.*, **135**, 761 (1940)
- (5) Balls, A. K., Thompson, R. R., and Kies, M.

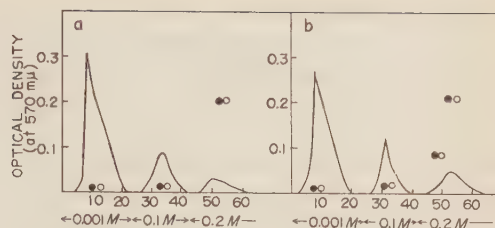


FIG. 12. Column chromatograms of bromelain from Amamioshima (a) and from Formosa (b) with DEAE-cellulose.

(0.9×20 cm., phosphate buffer, pH 6.8)

●, milk clotting ○, BAA hydrolysis

The activities are shown on arbitrary scales.

- W., *Ind. Eng. Chem.*, **33**, 950 (1941)
- (6) Masuda, T., *J. Biochem.*, **46**, 1489, 1569 (1959)
- (7) Heinicke, R. M., and Mori, R., *Science*, **129**, 3364 (1959)
- (8) Murachi, T., and Neurath, H., *J. Biol. Chem.*, **235**, 99 (1960)
- (9) Fu, T. H., *Yamaguchi-Igaku*, **9**, 1353 (1960)
- (10) Flodin, P., and Kupke, D. W., *Biochim. et Biophys. Acta*, **21**, 368 (1956)
- (11) Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 751 (1956)
- (12) Tiselius, A., Hjertin, S., and Levin, Ö., *Arch. Biochem. Biophys.*, **65**, 132 (1956)
- (13) Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929)
- (14) Conway, E. G., "Microdiffusion Analysis and Volumetric Error", G. Lockwood and Son Ltd., London, (1950)
- (15) Rosen, H., *Arch. Biochem. Biophys.*, **67**, 10 (1957)
- (16) Hestrin, S., *J. Biol. Chem.*, **180**, 249 (1949)
- (17) Korenman, I. M. and Ganichev, P. A., *Referat. Zhur, Khim.*, No. 29342 (1954), cit. from Chem. Abst. **49**, 3735 (1955)
- (18) Hirs, C. H. W., Moore, S., and Stein, H., *J. Biol. Chem.*, **200**, 493 (1953)
- (19) Ellis, S., and Simpson, M. E., *J. Biol. Chem.*, **220**, 939 (1956)
- (20) Doherty, D. G., and Popenoe, E. A., *J. Biol. Chem.*, **189**, 447 (1951)
- (21) Kitagawa, K., and Izumiya, N., *J. Biochem.*, **46**, 1159 (1959)
- (22) Izumiya, N., Okasaki, H., Matsumoto, I., and Takiguchi, H., *J. Biochem.*, **46**, 1347 (1959)
- (23) Smith, E. L., *Advances in Enzymol.*, **19**, 267 (1957)

Effect of X-ray Irradiation on the Function of Hemoglobin

By AKIHIKO KAJITA, AKIO HASHIMOTO, TARO OKAZAKI and
KOOZOO KAZIRO

(From the Department of Biochemistry, Nippon Medical School, Tokyo)

(Received for publication, February 17, 1961)

Since Fricke and Peterson (1) have found that hemoglobin was converted to methemoglobin by X-ray irradiation, much attention has been paid for the oxidation or decomposition of heme moiety of hemoglobin, by using large dose irradiation (2-4). However, very little is known about the effect of irradiation on the function of hemoglobin.

It is generally accepted that sulfhydryl enzymes have high susceptibility to ionizing radiations and are often inactivated by small dose irradiation (5-7). Hemoglobins are known to have sulfhydryl groups in their molecules which play an important role in the oxygen equilibrium (8-9) and in the oxidase activity (10). It has also been revealed that the functions of hemoglobin easily change by the treatment with *p*-chloromercuribenzoate (PCMB) (8-10), sodium benzoate (11), urea (12) *etc.* without any detectable change in the absorption spectra. Therefore, it may well be expected that when hemoglobin is irradiated in less doses than oxidation or decomposition of the molecule occurs, the functions of hemoglobin may alter even without any significant change in the absorption spectra. The authors intended to study the effect of X-ray irradiation on ethylisocyanide (EIC) equilibrium of hemoglobin which is comparable to the oxygen equilibrium of the pigment (12). The equilibrium system with EIC can be studied even with possible contamination of methemoglobin.

EXPERIMENTAL

Materials—a) Horse oxyhemoglobin was crystallized by the Heidelberger's method with slight modification by one of the authors (13). The crystals

were washed several times by chilled water until the clear red supernatant showed no catalase activity and were finally dissolved in 0.1 *M* phosphate buffer (pH 6.5) except where noted otherwise. This crystalline preparation was stored in an ice-box and was used for the experiment within a week.

b) Horse methemoglobin was prepared from oxyhemoglobin solution by addition of potassium ferricyanide and dialyzed against distilled water in a refrigerator until ferricyanide was completely removed.

c) Crystalline horse metmyoglobin was prepared from horse heart muscle by the method of Tsushima *et al.* (14). The crystal, collected by centrifugation, was dissolved in distilled water and dialyzed against water with stirring at cold, until no ammonium sulphate was detected.

d) Human blood catalase was crystallized four times after Herbert and Pinsent (15). The crystal was dissolved in water and dialyzed completely. Kat f of this preparation was 42,000.

e) Ethylisocyanide (EIC) was synthesized from ethyl iodide and silver cyanide by the method of Ga'utier (16) and stored at -20°C before use.

f) Reduced glutathione was furnished by Kirin Beer Co. Ltd. *p*-Chloromercuribenzoate (PCMB) was purchased from Daiichi Kagaku Co. Ltd. These preparations were used without further purification. All other chemicals were reagent grade materials.

Experimental Methods—a) X-ray irradiations were performed by the use of KXC 18 X-ray machine of Tōshiba Co. Ltd. of 150 kv peak, 5 mA, with a 5 mm. Al and a 10 mm. Cu filter. One hundred ml. of 7×10^{-6} *M* oxyhemoglobin solution was irradiated at a dose rate of 250 r/min. aerobically in an ice bath at 0°C .

b) Photo-oxidation was performed according to Weil *et al.* (17). One hundred ml. of 7×10^{-6} *M* oxyhemoglobin solution was illuminated by 200 W tungsten lamp at a distance of 20 cm. in the presence of 2.67 mg./dl. methylene blue, aerobically. The temperature of the solution was approximately 22°C throughout the illumination.

c) EIC binding reactions of hemoglobin and myoglobin were carried out at room temperature of 22–25°C. The irradiated hemoglobin solution was diluted to $4.67 \times 10^{-6} M$. To 10 ml. of this solution 5 ml. of appropriate concentration of EIC solution was added. An aliquot of this mixture was reduced by a minimum amount of sodium dithionite powder in a cuvette and covered with liquid paraffin.

d) Concentration of hemoglobin and myoglobin were estimated by alkaline denatured globin hemochrome method (18) and cyanide-metmyoglobin method (19) and were expressed in terms of their hematin content.

e) Optical determinations were made at room temperature of 22–25°C by use of EPV Model II photoelectric spectrophotometer of Hitachi Co. Ltd. and a cuvette of 10 mm. depth.

f) Hydrogen ion concentration was estimated by a glass electrode pH meter of Toyo Rika Co. Ltd.

RESULTS

Binding Reaction of EIC with Hemoglobin—

When EIC was added to reduced hemoglobin, the absorption spectrum was converted to that of EIC-hemoglobin (12). In the present study,

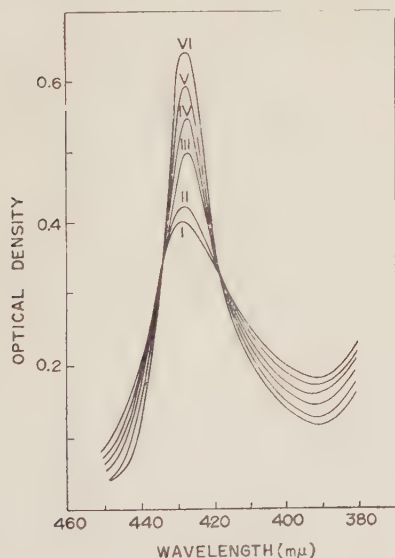


FIG. 1. Change of absorption spectra of hemoglobin by EIC binding.

Concentration of hemoglobin: $3.11 \times 10^{-6} M$
 Concentration of added EIC: I. 0 M, II. $3.1 \times 10^{-5} M$, III. $1.2 \times 10^{-4} M$, IV. $2.5 \times 10^{-4} M$, V. $6.1 \times 10^{-4} M$, VI. $2.5 \times 10^{-3} M$ and $3.1 \times 10^{-3} M$.
 pH 6.5.

the absorption change of hemoglobin by EIC addition was measured in the region of Soret band. Fig. 1 shows this change at pH 6.5. The presence of isosbestic points at 433 and 420 mμ throughout the absorption change indicates that the reaction system is composed of two components optically. The fractional saturation of EIC for hemoglobin (Y) was calculated from the optical change at 428 mμ, where the difference was maximum, using following equation:

$$Y = \frac{E_x - E_o}{E - E_o}$$

where E_x is the extinction measured, and E_o and E are the optical density of reduced hemoglobin and EIC-hemoglobin at 428 mμ, respectively.

As may be seen from the curve I of Fig. 2, the plot of Y against logarithmic concentration of free EIC (not combined to hemoglobin) gives a symmetric sigmoid curve. The observed values are in good agreement with a theoretical equilibrium curve of $n=2.4$ in Hill's equation (20):

$$Y = \frac{Kp^n}{1 + Kp^n}$$

K, p and n represent equilibrium constant, oxygen pressure (free EIC concentration in this case) and sigmoid coefficient, respectively.

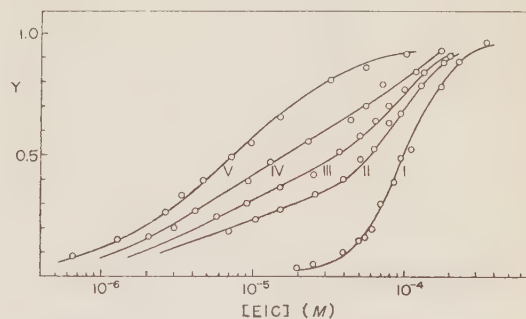


FIG. 2. Change of EIC equilibrium curve caused by X-ray irradiation.

Concentration of hemoglobin at irradiation: $7 \times 10^{-6} M$. Radiation doses: I. 0 r (0), II. 1,400 r (0.4), III. 2,800 r (0.55), IV. 4,200 r (0.75), V. 6,720 r (1.0) and 8,400 r (1.0).

Numbers in parenthesis stand for the ratios of modified hemoglobin to total hemoglobin (x).
 pH 6.5.

Effect of X-ray Irradiation on the Soret Band of Hemoglobin—On X-ray irradiation, the optical density at $414\text{ m}\mu$ of oxyhemoglobin decreased in parallel with irradiation doses, as previously reported by Barron *et al.* (4). However, the optical density at $430\text{ m}\mu$ of reduced hemoglobin also decreased, when measured after reduction with sodium dithionite, as may be seen from Fig. 3. Since

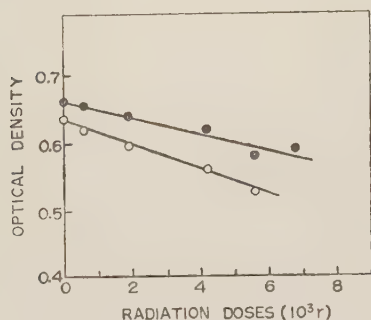


FIG. 3. Effect of X-ray irradiation on the Soret band of hemoglobin.

—●— Oxyhemoglobin ($414\text{ m}\mu$)

—○— Reduced hemoglobin ($430\text{ m}\mu$)

Hemoglobin concentration at irradiation:

$7 \times 10^{-6}\text{ M}$. Hemoglobin concentration at estimation:

$5 \times 10^{-6}\text{ M}$, pH 6.5.

methemoglobin should be reduced to hemoglobin by addition of sodium dithionite, these facts suggest that hemoglobin was oxidized not only to methemoglobin but also partially to an unknown breakdown product by irradiation. The decomposition of hemoglobin reached 9 per cent at a radiation dose of 4200 r. However, when EIC was added to this irradiated hemoglobin, the change of absorption spectrum was almost similar to those of non-irradiated hemoglobin, although two isosbestic points at 433 and $420\text{ m}\mu$ were shifted about $1\text{ m}\mu$ to longer and shorter wavelength respectively on irradiation of 6720 r. Therefore, the EIC equilibrium of irradiated hemoglobin, could be studied similarly to and compared with that of native hemoglobin. The results are summarized in Fig. 2. This figure shows that the EIC equilibrium curve of hemoglobin gradually shifted

to the left with the increase of radiation doses, and that the curve became assymmetric as well. With a radiation dose of about 6,000 r, the curve became symmetric again, the sigmoid coefficient being then $n=1.0$. Any change of the equilibrium curve was not observed by further irradiation.

It is obvious from this fact that heme-heme interaction of hemoglobin was completely lost and at the same time EIC affinity of hemoglobin increased intensively from 1×10^{-4} to 8×10^{-6} in Y_{50} (half saturated EIC concentration), on irradiation of about 6,000 r. As shown in Fig. 4, hemoglobin which had been

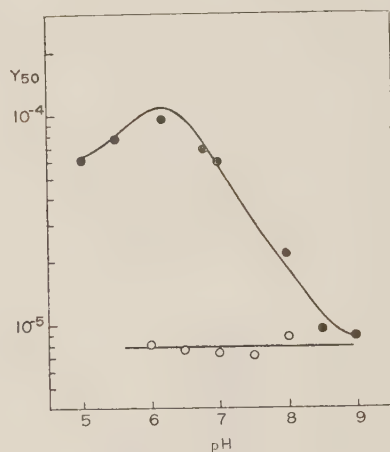


FIG. 4. pH dependence of Y_{50} in EIC binding reaction.

—●— Native hemoglobin

—○— Irradiated hemoglobin (irradiation was carried out at pH 6.5)

Hemoglobin concentration at irradiation:

$7 \times 10^{-6}\text{ M}$. Radiation dose: 7,200 r.

irradiated to this ultimate state, showed no pH dependence of Y_{50} in EIC binding reaction which is well known in the native hemoglobin as Bohr effect (21). The same fact has been reported by one of the authors (12) on urea denaturation of horse hemoglobin. It seems thus to be most probable that the change in EIC equilibrium curve of X-ray irradiated hemoglobin is due to the modification of protein moiety of the pigment, but not of the prosthetic group.

Assuming that the modification of globin

by irradiation arises from a single reaction without any intermediate step, concerning EIC equilibrium at least, it can be deduced that the asymmetry and the leftward shift of equilibrium curve are resulted from the concomitant presence of native and modified hemoglobins. Hence, the EIC equilibrium curve of a mixture of native and modified hemoglobin may be represented theoretically, as follows:

$$Y = \frac{K_1[\text{EIC}]^{2.4}}{1 + K_1[\text{EIC}]^{2.4}} \cdot (1-x) + \frac{K_2[\text{EIC}]}{1 + K_2[\text{EIC}]} \cdot x \dots 1$$

where, K_1 and K_2 are the equilibrium constants of the EIC binding reaction of native and modified hemoglobins, which are calculated to be 3.98×10^9 and 1.25×10^5 from $Y_{50}^1 = 1.0 \times 10^{-4}$ and $Y_{50}^2 = 8.0 \times 10^{-6}$ of curves I and V in Fig. 2, respectively. 'x' denotes the ratio of modified hemoglobin to total hemoglobin. Full circles in Fig. 2 represent the estimated values at a definite radiation doses, and coincided well with the theoretical curves (full lines), calculated from equation 1.

This fact strongly support the above assumption, that is, native hemoglobin may be converted to a modified state by irradiation with one step reaction, at least with respect to EIC binding properties.

Fig. 5 shows the relation of radiation doses and the modification per cent of hemoglobin by irradiation. The modification per cent is calculated by following equation:

$$\text{Modification \%} = \frac{x-a}{1-a} \times 100$$

where 'x' and 'a' stand for the ratios of modified hemoglobin to total hemoglobin in irradiated and non-irradiated samples. In dilute solution, hemoglobin is easily modified, even at 4–5°C in a refrigerator. As may be seen in Fig. 6, the EIC equilibrium curve of hemoglobin became asymmetric by aging and was shifted to the left as well as by X-ray irradiation. Similar phenomenon is observed in oxygen equilibrium of hemoglobin by Kubo *et al.* (22) and Takashima (23). Therefore, the preexisting amount of the modified hemoglobin by aging should be subtracted from the observed value in order

to calculate the modification per cent by irradiation.

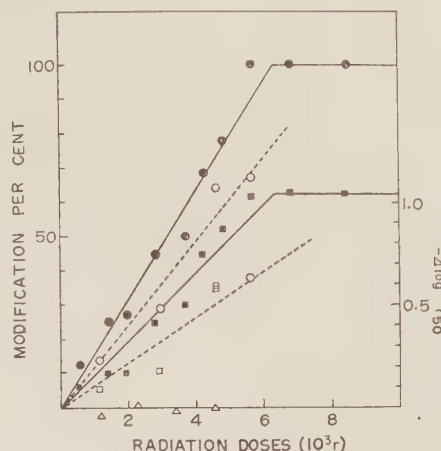


Fig. 5. Relations of the modification per cent to radiation doses.

Modification % { ● — Hemoglobin
○ — Methemoglobin
—Δ log Y_{50} { ■ — Hemoglobin
□ — Methemoglobin
△ — Metmyoglobin

Total hemoglobin concentration at irradiation : $7 \times 10^{-6} M$, pH at irradiation : 6.5.

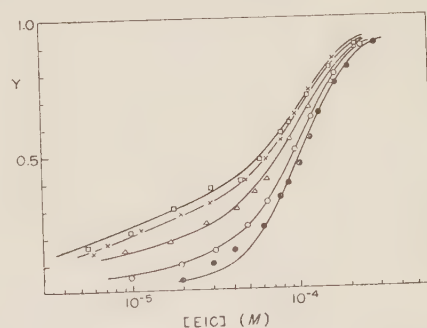


Fig. 6. Effect of aging upon the EIC equilibrium of hemoglobin.

—●— 0 time (0), —○— 10 hours (0.1),
—△— 2 days (0.25), —×— 9 days (0.35),
—□— 13 days (0.4), at 4°C.

Numbers in parenthesis stand for the ratios of modified hemoglobin to total hemoglobin.

Concentration of hemoglobin : $7 \times 10^{-6} M$, pH 6.5.

Modification per cent thus obtained were plotted against radiation doses. The modification per cent increased almost linearly up

to 6,000 r and did not increase by further irradiation.

Representing the increase of EIC affinity of hemoglobin by $-\Delta \log Y_{50}$, which is the difference of $\log Y_{50}$ between the irradiated sample and the control, the affinity was proved to be augmented by irradiation, as may be seen from Fig. 5.

As shown in Fig. 5, when methemoglobin was irradiated, the modification per cent and EIC affinity of the pigment in reduced state also rised as in the case of hemoglobin, although the rise was relatively small.

Myoglobin also combined with EIC, as previously reported (12). The sigmoid coefficient of equilibrium curve, n , was 1.0 and Y_{50} was 8.0×10^{-6} in the present study. These facts show that myoglobin has no heme-heme interaction and has a high affinity for EIC. X-ray denatured hemoglobin completely coincided with native myoglobin in these respects. As illustrated in Fig. 7, the EIC

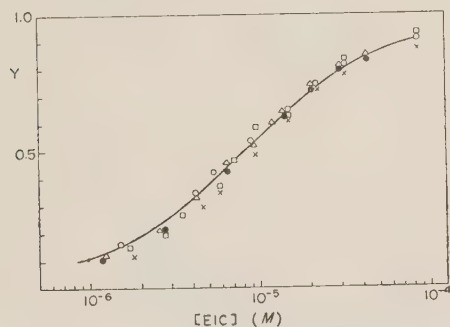


FIG. 7. Effect of X-ray irradiation on the EIC equilibrium curve of myoglobin.

○: control, ×: 1,134 r, □: 2,268 r, ●: 3,402 r, △: 4,536 r,

Concentration of metmyoglobin at irradiation: $7 \times 10^{-6} M$, pH 6.5.

equilibrium curve of myoglobin obtained by reduction of irradiated metmyoglobin was the same to that of native myoglobin (refer also to $-\Delta \log Y_{50}$ in Fig. 5).

Effect of Hemoglobin Concentration—After hemoglobin was irradiated at various concentrations, the solutions were diluted to the same concentration as above. Fig. 8 shows the results obtained at constant radiation

doses. The modification per cent and $-\Delta \log Y_{50}$ of hemoglobin decreased almost exponentially, in proportion to the concentration of hemoglobin.

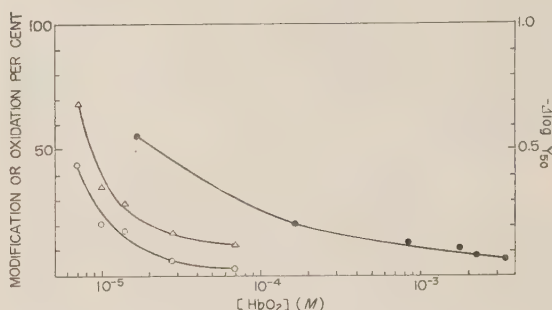


FIG. 8. Dependence of radiation effect on hemoglobin concentration.

—△— Modification % { 4,200 r, pH 6.5

—○— $-\Delta \log Y_{50}$

—●— Oxidation %: 50,000 r, pH 7.0 (Barron *et al.* (4)).

Effect of Hydrogen Ion Concentration—Hemoglobin was irradiated at various pH. The resulted solutions were adjusted to pH 6.5 by addition of appropriate concentration of HCl or NaOH solutions and diluted to previously mentioned concentration. The results were shown in Fig. 9. The modification per cent

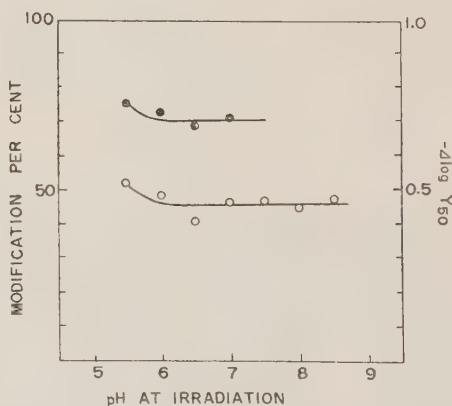


FIG. 9. Effect of pH at irradiation on modification of hemoglobin.

—○— Modification per cent, —●— $-\Delta \log Y_{50}$

Hemoglobin concentration at irradiation:

$7 \times 10^{-6} M$. Radiation doses: 4,200 r.

EIC binding reactions were carried out after pH of irradiated samples was adjusted to 6.5.

and $-\Delta \log Y_{50}$ were independent of pH in the range of pH 5.5–8.0. Slight augmentations tend to occur in more acidic range.

Effect of Protective Substances—When hemoglobin was irradiated in the presence of some protective substances, the change of EIC equilibrium curve reduced remarkably. Representing protection per cent (or restoration per cent) by P, it can be calculated from following equation:

$$P = \frac{A-B}{A} \times 100 (\%)$$

where A and B are the modification per cent of irradiated samples in the absence and presence of additions, respectively. The results obtained are listed in Table I.

Addition of catalase protected hemoglobin from irradiation by 20 per cent. This value suggests the degree of participation of hydrogen peroxide upon the modification of hemoglobin by irradiation, which is generated from water. Reduced glutathione when added

before irradiation completely protected hemoglobin, whereas after irradiation it restored the modification of hemoglobin by only 41 per cent.

It is well known that heme-heme interaction of hemoglobin reduced markedly on addition of 2 moles of PCMB per mole of hemoglobin and reversed on addition of reduced glutathione (5). Thus, hemoglobin was first irradiated in the presence of PCMB, blocking the sulfhydryl groups of hemoglobin and thereafter, reduced glutathione was added to this solution in order to release the blockade. The protection per cent of PCMB for radiation effect was calculated to be 69 per cent.

Decomposition of Irradiated Hemoglobin by Coupled Oxidation—Hemoglobin is known to decompose autocatalytically to verdohemoglobin through an intermediate choleglobin in the presence of ascorbic acid and molecular oxygen, as reported by Kaziro *et al.*

TABLE I
Effect of Protective Substances

Addition		Concentration at irradiation	Ratio ¹⁾ of modification		Modification % by irradiation ²⁾	Protection or Restoration ³⁾
			non-irrad.	irrad.		
None		—	20	75	70 (A)	—
Catalase		2.5 mg./dl.	10	60	56 (B)	20
Reduced glutathione	Before irradiation	$6.3 \times 10^{-3} M$	0	0	0 (B)	100
	After irradiation	$6.3 \times 10^{-3} M$	15	15	41	41
PCMB		$3.6 \times 10^{-6} M$	10	10	22 (B)	69
+ Reduced glutathione		$6.3 \times 10^{-3} M^{4)}$				

Concentration of hemoglobin at irradiation: $7 \times 10^{-6} M$.

Radiation doses: 4,200 r, pH 6.5

1) Ratio of modification = x

2) Modification % by irradiation = $\frac{x-a}{1-a} \times 100$

3) Protection or Restoration % = $\frac{A-B}{A} \times 100$

4) Reduced glutathione was added before and after irradiation.

(24). In this reaction system, the addition of PCMB accelerated the decomposition of hemoglobin, owing to the increase of oxidase activity (10). Therefore, it is anticipated that the decomposition rate of hemoglobin may increase by irradiation, because the sulfhydryl groups seem to play a role in the modification of hemoglobin on irradiation. The decomposition rate of irradiated hemoglobin was estimated by the decrease of optical density at $425\text{ m}\mu$, which is a peak of the Soret band of alkaline denatured globin hemochrome (Fig. 10). However, no signifi-

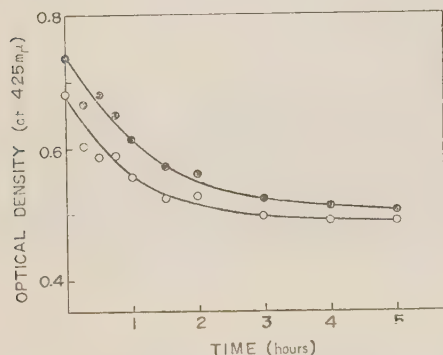


FIG. 10. Effect of X-ray irradiation on the decomposition rate of oxyhemoglobin.

Oxyhemoglobin: $7 \times 10^{-6} M$, 4.9 ml. (pH 6.5): Ascorbic acid (neutralized) $2.5 \times 10^{-2} M$, 0.1 ml.

The reaction mixture was incubated at $37^\circ C$. At appropriate time interval, the reaction was stopped by addition of 2.5 ml. of 4% NaOH and a minimum amount of $Na_2S_2O_4$. After standing for 30 minutes at room temperature, optical density at $425\text{ m}\mu$ was estimated.

—●— Control, —○— Irradiated (4,200 r)

cant difference of the decomposition rate between irradiated and non-irradiated hemoglobin was detected.

Photo-oxidation—Hemoglobin was photo-oxidized after Weil *et al.* (17) in the presence of methylene blue. The EIC equilibrium curve of oxidized samples were illustrated in Fig. 11. The results show the decrease of heme-heme interaction and increase of EIC affinity as in the case of X-ray irradiation.

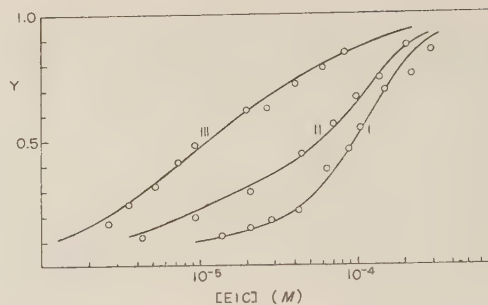


FIG. 11. Effect of photo-oxidation on the EIC equilibrium curve of hemoglobin.

100 ml. of $7 \times 10^{-6} M$ oxyhemoglobin solutions were illuminated by 200 W tungsten lamp at a distance of 20 cm. in the presence of 2.67 mg. methylene blue, aerobically. Illumination time: I; control (0.2), II; 1 hour (0.45), III; 3 hour (0.9).

Numbers in parenthesis stand for the ratios of modified hemoglobin to total hemoglobin.

DISCUSSION

It is generally accepted that the action of ionizing radiation on enzyme or protein in solution is due to the secondary effect of free radicals and peroxide which are generated from water molecule. Formation of methemoglobin and unknown green product from hemoglobin on irradiation were also believed to be caused by the action of these oxidizing groups (1-4). As shown in Fig. 3, slight decrease of optical density of oxyhemoglobin at $414\text{ m}\mu$ was observed on irradiation. Similar results were obtained, when the irradiated samples were reduced by sodium dithionite. The optical density at $430\text{ m}\mu$ of reduced hemoglobin also decreased, as may be seen from Fig. 3. This fact suggests that oxyhemoglobin was oxidized not only to methemoglobin but also to an unknown breakdown product to some extent by irradiation, because methemoglobin should be quantitatively reduced to hemoglobin by sodium dithionite. Barron *et al.* (4) reported that the optical density of oxyhemoglobin at $414\text{ m}\mu$ decreased by 10.17 per cent and 94.2 per cent on irradiation of 10,000 r and 100,000 r, respectively. In the present study, the decrease of 10.6 per cent was observed on irradiation of 6,720 r. On the other hand, the function

of hemoglobin in combining EIC was changed maximally, even with such a relatively small change in the absorption spectrum. In this state, the heme-heme interaction and the Bohr effect of hemoglobin has completely disappeared, whereas EIC affinity augmented notably. It seems to be of great interest, that this modified hemoglobin possesses quite similar characteristics to myoglobin in respect to their function in combining EIC. However, whether hemoglobin molecule dissociated to subunits or not, remains obscure. Similar results have been obtained on hemoglobin solution with urea (12) or mersalyl (8). Therefore, above mentioned functional change of hemoglobin is apparently due to the modification of its globin moiety, though the possible dissociation of the molecule to subunits being unexcluded. On the other hand, there exist also some hemoglobin molecules in which hemes were oxidized or decomposed, even on an irradiation below 6,720 r, the amount of which, however, can safely be neglected in the present study. Isosbestic points could clearly be demonstrated in the absorption change of irradiated hemoglobin following with EIC combination. The authors presented the equation 1 theoretically, assuming that hemoglobin undergoes some kind of modifications on the globin moiety by X-ray irradiation. The results obtained were in good agreement with the theoretical curve derived from this equation. The assumption may be supported by an analogous observation in experiments on the irradiation of methemoglobin.

In general, sulfhydryl enzymes have high sensibility to ionizing radiations. Hemoglobins are well known to have sulfhydryl groups in their molecules. When the sulfhydryl groups of hemoglobin are blocked by PCMB etc., the function of hemoglobin changes profoundly (8-10). It is presumed from these facts the functional modification of hemoglobin by irradiation may be a result of an oxidation of sulfhydryl groups by free radicals and peroxide which are generated from water molecule. In fact, metmyoglobin which has no sulfhydryl groups, did not show any

significant change in EIC equilibrium on irradiation (Fig. 5 and 7). Addition of reduced glutathione before irradiation completely protected hemoglobin from radiation effect (Table I). These facts support the validity of above mentioned presumption. By Dale *et al.* (25) the protective action of reduced glutathione or cysteine on catalase from irradiation was assumed to be due to the formation of inactive complexes with hematin. In the case of hemoglobin, however, reduced glutathione seems to protect the oxidation of sulfhydryl groups in globin molecule, because sulfhydryl compounds cannot combine with ferrous heme. Addition of reduced glutathione after irradiation restored the activity of hemoglobin by only 41 per cent, differing from that of before irradiation. The fact implies that addition of glutathione after irradiation restored the function of hemoglobin only partly by reducing the oxidized sulfhydryl groups in globin molecule. Apparently, the secondary modification of hemoglobin structure, which is resulted from the oxidation of sulfhydryl groups, cannot be restored by glutathione. It was also revealed that the addition of PCMB protected hemoglobin from irradiation by 69 per cent, blocking two sulfhydryl groups per one molecule of hemoglobin. These results suggest that the oxidation of sulfhydryl groups in hemoglobin molecule play an important role for the modification of hemoglobin by X-ray irradiation.

It should be noted, however, that other amino acid residues than sulfhydryl groups can also be attacked by free radicals or peroxide on ionizing radiation. For example, imidazole nucleus of histidine is known to decompose with high ionic yields by irradiation (26, 27). Therefore, the authors investigated the function of photo-oxidized hemoglobin, in which imidazole nucleus of histidine is decomposed. The results obtained were quite similar to those of X-ray irradiated hemoglobin. Consequently, the decomposition of histidine may possibly be an additional factor of hemoglobin modification on X-ray irradiation. However, this assumption seems

to have less validity, in consideration of the fact that metmyoglobin molecule was not affected by X-ray irradiation, although the molecule contains histidine residues.

From the experiment on coupled oxidation of hemoglobin with ascorbic acid, it is clear that the modification of hemoglobin on irradiation is not caused merely by oxidation of sulfhydryl groups in the molecule. The decomposition rate of X-ray irradiated hemoglobin in this reaction showed no significant difference with non-irradiated hemoglobin (Fig. 11). From all these facts, it is deduced most probably that the modification of hemoglobin by X-ray irradiation is caused mainly and primarily by oxidation of sulfhydryl groups in the globin molecule and followed by the secondary modification of protein conformation.

SUMMARY

Horse hemoglobin was irradiated by X-ray. Ethylisocyanide (EIC) binding reaction of irradiated hemoglobin was investigated.

1. From the EIC equilibrium, it was elucidated that the heme-heme interaction and the Bohr effect of hemoglobin decreased remarkably, whereas EIC affinity increased intensively on irradiation.

2. Radiation effect increased in proportion to the radiation doses, the modification of hemoglobin being at its maximum state on the radiation doses exceeding 6,000 r. In this state, the function of hemoglobin was quite similar to that of myoglobin which has no heme-heme interaction, no Bohr effect, and a high EIC affinity.

3. These changes in the EIC binding function of hemoglobin seem to be due to the modification of the globin moiety. The observed leftward shift and the asymmetric figure of the EIC equilibrium curve was explained by the mixed system of two components, native and modified hemoglobin. The theoretical equation for EIC equilibrium curve of a mixed system was proposed. The results obtained were in good agreement with the theoretical curve.

5. Myoglobin, when irradiated in its

ferric state, showed no change on the EIC equilibrium curve as compared with that of native myoglobin, whereas irradiated methemoglobin gave a similar change as in the case of oxyhemoglobin.

6. Catalase protected hemoglobin from irradiation by 20 per cent. Addition of glutathione before irradiation protected hemoglobin completely, although after irradiation restored only by 41 per cent. PCMB protected hemoglobin by 61 per cent.

7. Irradiation did not accelerate the decomposition rate of hemoglobin to verdohemoglobin.

8. The possible role of sulfhydryl groups on the modification of hemoglobin by irradiation was discussed from these results.

The authors wish to express their cordial thanks to Dr. H. Matsudaira, Department of Radiology, School of Medicine, Tokyo University, for technical assistance on X-ray irradiation and to Dr. H. Hirai, Department of Biochemistry, School of Medicine, Tokyo University, for a gift of crude crystall of human erythrocyte catalase. Thanks are also due to the Scientific Research Fund of the Ministry of Education. The essential part of this work was published in Japanese (28).

REFERENCES

- (1) Fricke, H., and Peterson, B. W., *Am. J. Roentogenol.*, **17**, 611 (1927)
- (2) Laser, H., *Nature*, **176**, 361 (1955)
- (3) Dowben, R. M., and Walker, J. K., *Proc. Soc. Exptl. Biol. Med.*, **90**, 398 (1955)
- (4) Barron, E. S. G., and Johnson, P., *Rad. Research*, **5**, 290 (1956)
- (5) Barron, E. S. G., Dickman, S., Munts, J. H., and Singer, T. P., *J. Gen. Physiol.*, **32**, 537 (1949)
- (6) Barron, E. S. G., and Dickman, S., *J. Gen. Physiol.*, **32**, 595 (1949)
- (7) Tanaka, S., Hatano H., and Ganno, S., *J. Biochem.*, **46**, 485, 925 (1959); *ibid.*, **47**, 361 (1960)
- (8) Riggs, A. F., *J. Gen. Physiol.*, **36**, 1 (1952)
- (9) Riggs, A. F., and Wolbach, R. A., *J. Gen. Physiol.*, **39**, 585 (1956)
- (10) Kajita, A., *J. Japan. Biochem. Soc.*, **28**, 290 (1956)
- (11) Kikuchi, G., and Tomimura, T., *J. Biochem.*, **41**, 503 (1954)
- (12) Okazaki, T., and Tsushima, K., *J. Biochem.*,

- 46**, 433 (1959)
- (13) Suzuki, M., Kajita, A., and Hanaoka, C., *J. Biochem.*, **41**, 401 (1954)
- (14) Tsushima, K., and Okazaki, T., *J. Japan. Biochem. Soc.*, **26**, 232 (1954)
- (15) Herbert, D., and Pinsent, J., *Biochem. J.*, **43**, 203 (1948)
- (16) Gautier, A., *Ann. Chim. Phys.*, **17**, 215 (1869)
- (17) Weil, L., Buchert, A. R., and Maher, J., *Arch. Biochem. Biophys.*, **40**, 245 (1952)
- (18) Drabkin, D. L., *J. Biol. Chem.*, **146**, 605 (1942)
- (19) Drabkin, D. L., *J. Biol. Chem.*, **171**, 409 (1947)
- (20) Hill, A. V., *J. Physiol. London*, **40**, 4p. (1910)
- (21) Bohr, C., Hasselbalch, U., and Krogh, A., *Scand. Arch. Physiol.*, **16**, 402 (1904)
- (22) Kubo, M., *Bull. Chem. Soc. Japan*, **29**, 649 (1956)
- (23) Takashima, S., *J. Am. Chem. Soc.*, **77**, 6173 (1955)
- (24) Kaziro, K., Kikuchi, G., and Hanaoka, C., *J. Biochem.*, **42**, 423 (1955)
- (25) Dale, W. M., and Russel, C., *Biochem. J.*, **62**, 50 (1956)
- (26) Dale, W. M., Davies, J. V., and Gilbert, C. W., *Biochem. J.*, **45**, 93 (1949)
- (27) Bhatia, D. S., and Procter, B. E., *Biochem. J.*, **49**, 550 (1951)
- (28) Hashimoto, A., *J. Japan. Biochem. Soc.*, **32**, 485 (1960)

High-Voltage Paper Electrophoresis of Iodine-Containing Compounds in Thyroid Gland*

By SHO MIYAMOTO and TSUNEO KOBAYASHI

(From the Department of Biochemistry, Tokyo Medico-Dental University, and Japan Red Cross Central Hospital, Tokyo)

(Received for publication, February 28, 1961)

Advances in radioisotopic technique and paper chromatographic method brought about progress in the biochemistry of thyroid gland in recent years.

In these years, Miyamoto and his collaborators (1) improved the high-voltage paper electrophoresis (HVPE) apparatus of Heilmeyers perpendicular type (2) to the horizontal type and applied it to the separation of various kinds of biological substances of lower molecular weight.

One of the collaborators (Takahashi (3)) determined the relative mobility of those compounds in term of R_{Lys} (mobility ratio in respect to L-lysine) by running HVPE in acidic buffer (5% formic acid-15% acetic acid, pH at about 1.5). It was suspected later, however, whether or not a sole ninhydrin-positive band in acidic HVPE pattern of thyroxine actually represented undecomposed thyroxine itself, because its radioautogram showed only a faint band of I^{131} at the place of ninhydrin-positive band, but it produced a thick, intensive black band of I^{131} at a very low mobility. HVPE in acidic buffer seemed thus inadequate, at least, for thyroxine because of its possible degradation (4).

It seemed of interest, therefore, to run HVPE alternatly in alkaline buffer. The results of HVPE in borate buffer of pH 10.8, including the establishment of optimum pH, and radioautographic procedures by the aid of NaI^{131} for the determination of relative mobilities of thyroxine-related compounds, are presented in this paper.

* A part of this work was published in Geka-no-Ryoiki, 9, 1 (1960)

EXPERIMENTAL

Apparatus—A high-voltage paper electrophoresis apparatus, model FE 105 (Fuji Medical Instrument Co.) designed by Miyamoto *et al.* (1) was employed. This horizontal type of HVPE apparatus was proved not only better than the vertical type of Heilmeyer (2) in producing a highly equal potential field all over the filter paper but was found to have a more efficient cooling during HVPE through the coolant (*n*-hexane) than other horizontal type of instrument equipped with cooling coils.

Materials—Reagents: (a) Borate buffers, ranging from pH 8.6 to 11.9, were prepared by adding *N* sodium hydroxide to *M*/20 boric acid solution.

(b) Pauly's diazo reagents. Reagent I (diazotized sulfanilic acid) and reagent II (alcoholic sodium carbonate solution) were freshly prepared prior to spraying by the method of Leblond and Gross (5).

(c) Thyroxine and its related compounds.

(i) 3, 5-Diiodo-L-thyroxine (DIT) (Sigma chemical Co.).

(ii) 3, 5-Diiodo-DL-thyronine (T_2) (Aldrich Chemical Co.).

(iii) 3, 5, 3'-Triiodo-L-thyronine (T_3) (Aldrich Chemical Co.).

(iv) Thyroxine (3, 5, 3', 5'-tetraiodo-L-thyronine) sodium salt (T_4) (Byron Chemical Co.).

(v) DL-Thyronine (T_0) (British Drug House).

(vi) L-Thyroxine- U - I^{131} (Abbott Laboratories).

(vii) NaI - I^{131} solution (50 microC./ml.).

Methods—(A) *Paper Electrophoresis*: A set of 3 strips (2×69 cm.) of filter paper (Toyo Roshi No. 51 A) was used at each run of HVPE, in order to correct, if necessary, the mobility of the compound tested in the presence of NaI - I^{131} as the mobility standard. The paper strips were immersed in a borate buffer of desired pH, pressed gently between two sheets of dry filter paper to soak up an excess of buffer solution, and placed parallel on the plastic support

in the order of Nos. 1, 2 and 3. A solution of the compound to be tested was smeared by a glass capillary or a small brush along the starting line of paper strip No. 1 which was previously marked by pencil at 12 cm. from anodic end of the filter paper. A mixture of NaI-I^{131} solution and sample compound was placed on the paper strip No. 2. NaI-I^{131} solution was smeared by the same way on the paper strip No. 3.

A set of paper strips fixed to the support was then placed on the bottom of HVPE box filled with fresh coolant (*n*-hexane), both ends of each filter paper strips being dipped in the anodic and cathodic buffers in respective vessels. Usually, direct current of 3 kv. was applied for about 25 minutes at the coolant temperature of 2–4°C.

(B) *Identification of the Bands by Color Reaction:* After HVPE, the filter papers were dried and sprayed successively with Pauly's reagents I and II. Since the band of iodide was hardly located by the ceric sulfate-arsenite reagent, the following radioautographic procedure was necessary.

(C) *Radioautography:* A minute amount of NaI-I^{131} was smeared on both sides of the colored band of the test compound in paper strips Nos. 1 and 2. The three paper strips were laid on top of each other on non-screening film (Fuji Photo film Co.), and with the starting line and long sides in the same position, and left for 24 hours.

(D) *Calculation of R_I Values:* With the developed radioautogram, distances were measured from starting line to the iodide- I^{131} band, and also to the band of the sample compound marked by NaI-I^{131} .

The ratio of mobilities, taking iodide mobility as 1.0, is represented as the R_I value.

RESULTS

I. Indirect Determination of the R_I Values of Thyroxine at Various pHs—An example of

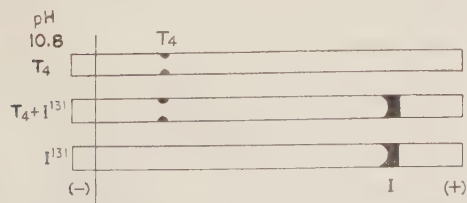


FIG. 1. Radioautogram of thyroxine following HVPE in borate buffer of pH 10.8.

radioautogram of thyroxine (T_4), as shown in Fig. 1, was prepared from a set of filter paper strips subjected to HVPE at pH 10.8.

In the same way, the radioautograms of T_4 were obtained for respective sets of HVPE pattern at 10 kinds of pH from 8.6 to 11.9. Radioautograms of each of the center paper strips (No. 2 as described in the part of Method) are summarized in Fig. 2 and represented as the R_I values in Table I. It is noted from Table I that R_I of T_4 is least variable at pH above 10.2.

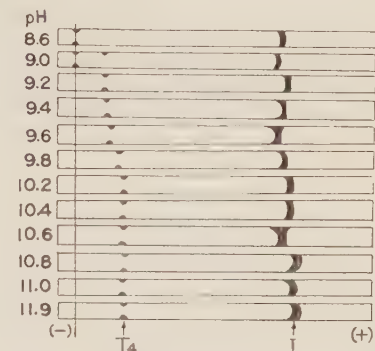


FIG. 2. Radioautograms of thyroxine following HVPE in borate buffers, ranging from pH 8.6 to 11.9.

II. *Direct Radioautographic Determination of R_I Values of I^{131} -labeled Thyroxine*—HVPE of $T_4\text{-U-I}^{131}$ was carried out in borate buffers, ranging from pH 9.0 to 11.9 and then autoradiographed without applying Pauly's reagents. As shown in an example (Fig. 3), R_I could be measured fairly accurately from

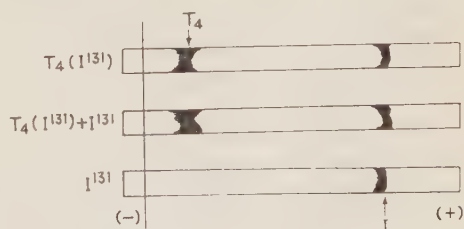


FIG. 3. Radioautogram of I^{131} -labeled thyroxine following HVPE in borate buffer of pH 10.8.

the direct radioautogram, in which the mobility of T_4 was also not influenced by coexisting iodide- I^{131} used as the mobility standard. The radioautograms of the center paper strips are illustrates in Fig. 4. The R_I values calculated are summarized in

TABLE I
*R_I Values of Unlabeled Thyroxine (T₄) Determined by HVPE
at Different Alkaline pHs*

pH	8.6	9.0	9.2	9.4	9.6	9.8	10.2	10.4	10.6	10.8	11.0	11.9
	0	0	0.13	0.16	0.18							
	0	0	0.13	0.16	0.18	0.22	0.23	0.22	0.23	0.24	0.23	0.23
	0	0	0.14	0.16	0.18	0.19	0.21	0.21	0.23	0.24	0.23	0.23
	0	0	0.14	0.16	0.18	0.22	0.24	0.26	0.26	0.23	0.27	0.24
	0	0	0.14	0.14	0.19	0.21	0.24	—	0.26	0.23	0.27	0.24
	—	—	0.13	—	—	—	0.25	—	0.26	—	0.26	0.24
Av.	0	0	0.14	0.16	0.18	0.21	0.23	0.23	0.25	0.24	0.25	0.24

TABLE II
*R_I Values of Uniformly I¹³¹-Labeled Thyroxine (T₄-U-I¹³¹) Determined
by HVPE at Different Alkaline pHs*

pH	9.0	9.2	9.4	9.6	9.8	10.2	10.4	10.6	10.8	11.0	11.9
	0	0.11	0.14	0.15	0.17	0.20	0.22	0.23	0.25	0.25	0.23
	0	0.13	0.15	0.15	0.18	0.20	0.22	0.22	0.23	0.23	0.23
	0	0.13	0.14	0.16	0.18	0.21	0.23	0.24	0.24	0.24	0.23
Av.	0	0.12	0.14	0.15	0.18	0.20	0.22	0.23	0.24	0.24	0.23

Table II. These direct and indirect *R_I* values are compared in Fig. 5.
The results presented above led to a conclusion that HVPE in borate buffer of pH

III. Indirect Determination of the *R_I* Values of Compounds Related to Thyroxine—By the same

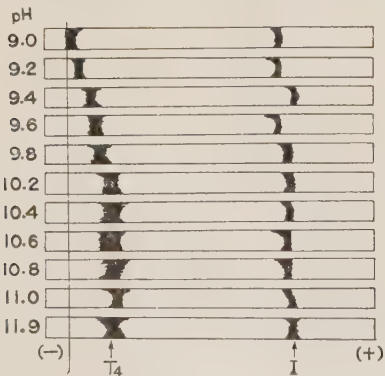


FIG. 4. Radioautograms of I¹³¹-labeled thyroxine following HVPE in borate buffers, ranging from pH 9.0 to 11.9.

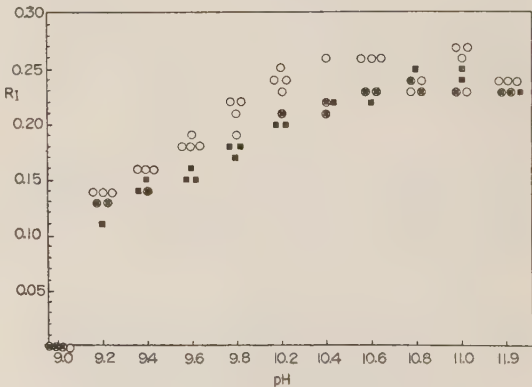


FIG. 5. The *R_I* values of thyroxine (○) and I¹³¹-labeled thyroxine (■) in alkaline pH (borate buffer).

For the details of determining the *R_I* values, see the text.

10.8 gave the most reproducible *R_I* values, at least, of T₄ without any possibility of deiodination as in the case of acidic HVPE (4).

procedures, the *R_I* values of T₀, T₂, T₃, and DIT were determined by running HVPE in borate buffer of pH 10.8. Reproducibility

was also examined by repeating each experiment, which are summarized in Table III.

TABLE III
*R_I Values of Thyroxine and Its Related Compounds
Determined by HVPE at pH 10.8*

	DIT	T ₀	T ₂	T ₃	T ₄
	0.38	0.40	0.26	0.29	0.24
	0.41	0.33	0.25	0.32	0.24
	0.40	0.32	0.27	0.31	0.23
	0.41	0.32	0.27	0.31	0.23
Av.	0.40	0.32	0.26	0.31	0.24

It is concluded that the R_I values of respective compounds in 4 runs are in a good agreement with each other, though the R_I values between T_4 and T_2 , and between T_3 and T_0 are considerably close to each other.

DISCUSSION

In this paper the optimum pH was experimentally determined for HVPE separation of T_4 and its related compounds. By use of iodide- I^{131} as the mobility standard the relative mobility in terms of R_I of respective compounds was quite reproducible at each pH, especially above pH 10.2. Thus, HVPE of T_4 in borate buffer of pH 10.8 not only gave the most agreeable R_I values in 4-6 runs but also left no possibility for degradation of T_4 during HVPE as shown by a close agreement of R_I values of T_4 determined by direct (labeled T_4) and indirect procedures (Fig. 5).

The optimum conditions established in HVPE experiments of T_4 also gave highly reproducible R_I values of DIT, T_0 , T_2 and T_3 , as shown in Table III. For the purpose of analyzing these compounds in the thyroid gland extract and other biological fluids, the alkaline HVPE may not be the perfect method by itself, since the R_I values were fairly close between T_4 and T_2 or between T_0 and T_3 (Table III) and since the band of DIT visualized by Pauly's reagents was rather broad to permit a highly accurate measurement of mobility. In this connection, the

results reported by Takahashi (3) on the relative mobility, R_{Lys} (L-lysine used as the mobility standard), of the compounds in thyroid gland are very useful to supplement the R_I values for the purpose of separation and identification of each compound. It is an advantage of acidic (pH 1.5) HVPE that the band of DIT developed by nin-hydrin reaction is far sharper than that in alkaline HVPE obtained by diazo reaction. R_{Lys} values of 5 compounds (Takahashi (3)) are summarized in Table IV in comparison with R_I values determined by the experiments in the present work.

TABLE IV
*Average Values of R_{Lys} and R_I of Thyroxine
and Its Related Compounds*

Compounds	R_{Lys}	R_I
DIT	0.25	0.40
T_0	0.36	0.32
T_2	0.21	0.26
T_3	0.08	0.31
T_4	2.24 ^{a)} (?)	0.24

a) This R_{Lys} value of T_4 was suspected to be that of deiodized or degraded T_4 (4) as explained in the text.

The HVPE procedures in both acidic and alkaline buffer have been applied to the thyroid gland extract of experimental animals given I^{131} to study the metabolism of thyroidal hormone (6, 7).

SUMMARY

1. The most reproducible conditions for HVPE in alkaline buffer of T_4 were established as borate buffer of pH 10.8 under a potential of 3 kv. for about 25 minutes.

2. A simple procedure was described for identifying I^{131} -labeled T_4 by use of HVPE of a mixture of T_4 with NaI- I^{131} , followed by radioautography. Mobility of iodide- I^{131} was taken as the standard and the relative mobility of T_4 -U- I^{131} was calculated as the R_I value.

3. In the case of unlabeled T_4 the T_4 -band visualized by diazo color reaction (Pauly's reagent) was marked by NaI- I^{131} ,

followed by radioautography as above.

4. The R_I value of T_4 (indirect method) agreed well with that of T_4 -U- I^{131} (direct method). This will exclude any possibility of deiodination or degradation of T_4 during HVPE at pH 10.8 which happened to be observed in HVPE of T_4 at pH 1.5 (4).

5. HVPE in borate buffer of pH 10.8 gave well reproducible R_I values of other 4 T_4 -related compounds, as follows: DIT 0.40 (0.38–0.41); T_0 0.32 (0.30–0.33); T_2 , 0.26 (0.25–0.27); T_3 , 0.31 (0.29–0.32); T_4 , 0.24 (0.23–0.24).

REFERENCES

(1) Miyamoto, S., Sakagishi, Y., Hirase, S., Ito,

Y., Bizen, R., Ashizawa, A., Nagata, I., and Morita, M., *J. Japan. Biochem. Soc.*, **29**, 731 (1958)

(2) Heilmeyer, L., Clotten, R., Sano, I., Strum, A., and Lipp, A., *Klin. Wschr.*, **32**, 831 (1954)

(3) Takahashi, T., *Geka-no-Ryoiki*, **7**, 1835 (1959); **8**, 71, 74 (1960)

(4) Futonaka, H., Mochizuki, A., and Komatsu, S., *Nippon Naibumpi Gakkai Zasshi*, **36**, 234 (1960); Mochizuki, A., and Takahashi, T., *Seibutsu Butsuri-Kagaku*, **6**, 203 (1960)

(5) Leblond, C. P., and Gross, J., *Endocrinol.*, **48**, 714 (1951)

(6) Naito, M., *Geka-no-Ryoiki*, in preparation

(7) Araki, Y., *Geka-no-Ryoiki*, in preparation

Inhibition of ATP-induced Contraction of Mitochondria by Polyhydroxylic Compounds*

By ALBERT L. LEHNINGER**

(From the Department of Physiological Chemistry, The Johns Hopkins School of Medicine, Baltimore 5, Maryland)

(Received for publication, April 12, 1961)

Sucrose in concentrations ordinarily used for isolation of mitochondria from homogenates of animal tissues ($\geq 0.25 M$) has been found to inhibit ATP-induced contraction of swollen rat liver mitochondria (1-5) and also to inhibit, although not as severely, the swelling of mitochondria induced by such agents as thyroxine (2, 6). These effects of sucrose are fully reversible (1). Since sucrose has also been found to inhibit the dinitrophenol-stimulated ATP-ase (7), the ATP- iP^{32} exchange reaction (8), and oxidative phosphorylation (9) as these reactions occur in the digitonin fragments of mitochondria membranes (10), it was postulated that the swelling-contraction cycle of mitochondria and the energy-coupling mechanism of oxidative phosphorylation share a common sucrose-inhibited reaction (1-5). A relationship between the mechanism of the mitochondrial swelling and contraction cycle and that of oxidative phosphorylation is supported by findings with other inhibitors (cf. (4, 5)).

Since sucrose would ordinarily not be considered as having the chemical potentialities of structure to act as an enzyme inhibitor, the specificity of these effects has been examined further and some attempts made to characterize the nature of the inhibition. It will be shown that inhibition of mitochondrial contraction is characteristic not only of sucrose but also of a large variety of polyhydroxylic compounds. However, it is not given by simple alcohols or diols.

* Supported by grants from the National Science Foundation, National Institutes of Health, the Nutrition Foundation, Inc. and the Whitehall Foundation.

** With the technical assistance of Mary Towart.

EXPERIMENTAL AND RESULTS

Measurements of the rates of mitochondrial swelling and contraction were carried out optically or gravimetrically as described before (1-3). Inhibitory effects on ATP-ase, ATP- iP^{32} exchange and the P:O ratio of digitonin fragments of rat liver mitochondria (11) were studied as described earlier (7, 10). Precise details are given in the legends.

Purity of Sucrose—After the inhibitory effect of sucrose on mitochondrial contraction was first observed (1), it was considered it might be caused by impurities in the samples employed, particularly because Dickens and Salmony (12) earlier had reported that ATP readily reversed thyroxine-induced swelling of mitochondria in a medium containing 0.3 M sucrose, a finding which we have never been able to confirm. While the reason for this discrepancy is still not understood, the inhibition does not appear to be caused by impurities in the sucrose. All commercial specimens tested have given inhibition of the same order of magnitude. Repeated recrystallization of sucrose, in the absence or presence of metal-chelating agents, did not remove the inhibitory action. Passage of sucrose solutions through columns of anion and cation exchangers likewise did not affect the inhibition of contraction. Ca^{++} , which is often an impurity in sucrose, has no inhibitory effect on contraction in concentrations to 0.01 M.

Inhibitory Effect of Other Sugars—A wide variety of other sugars and carbohydrates has been tested for their action on the contraction of thyroxine-swollen rat liver mitochondria in the presence of ATP + Mg^{++} + bovine serum

albumin, under conditions previously employed (3). In Fig. 1 is shown a comparison of the action of sucrose, which has been employed in this investigation as a reference standard, with that of D-glucose, D-xylose, and glycerol. In these tests the medium contained the solutes in the concentrations indicated, in addition to 0.02 *M* tris-HCl buffer pH 7.4. The normal controls contained 0.125 *M* KCl instead of the sugars. The media also contained 1.0×10^{-5} *M* L-thyroxine as swelling agent.

The findings summarized in Fig. 1 show that swelling of the mitochondria occurs in

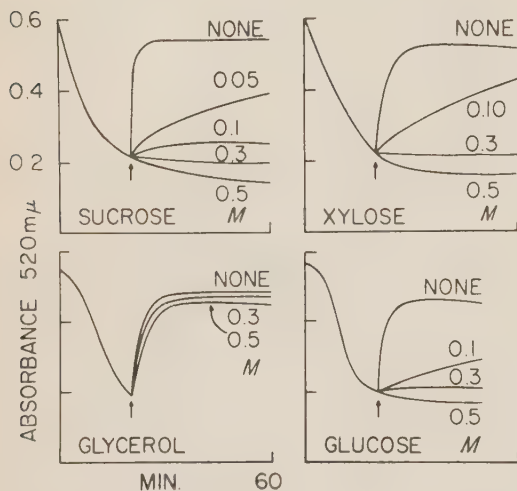


FIG. 1. Effect of some polyols on ATP-induced contraction of swollen rat liver mitochondria.

The actual data of selected experiments from Table I were normalized for swelling rate and extent and plotted to show the rate of ATP-induced contraction. The test system contained 5.0 ml. 0.02 *M* tris-HCl buffer pH 7.4 and 1×10^{-5} *M* L-thyroxine. The control tubes (labeled "NONE") contained also 0.125 *M* KCl and the experimental tubes the indicated concentrations of the polyols. At zero time a small aliquot of freshly prepared rat liver mitochondria was added to produce the indicated zero-time absorbancy at 520 *mμ* in 15 × 100 mm. tubes in a Beckman B spectrophotometer. The changes were followed at 20°C. At points designated by vertical arrows, a mixture of ATP, MgCl₂ and bovine serum albumin was added in a volume of 0.15 ml. and rapidly stirred, to yield final concentrations of 0.005 *M* ATP, 0.005 *M* MgCl₂, and 0.4 mg. per ml. albumin.

all of the media. On addition of ATP + Mg⁺⁺ + serum albumin, however, no optically demonstrable contraction of the mitochondria occurs in media containing 0.3 *M* sucrose, glucose, or xylose, whereas 0.3 *M* glycerol did not inhibit the contraction. At lower concentrations of the sugars some differences in potency of inhibition appear, xylose being least active.

In Table I are summarized the results of similar experiments with other sugars and polyhydroxylic compounds, with the inhibitory action of each compound at 0.3 *M* indicated semiquantitatively, as well as the approximate concentration giving 50% inhibition. On inspection of these data it is evident that substantial inhibition of contraction is given by a wide variety of polyhydroxylic compounds. These include simple pentoses such as D-xylose and D-ribose, 'hexoses' such as D-glucose, D-fructose, D-mannose and L-sorbose, 'disaccharides', including sucrose, lactose, maltose, the 'trisaccharide' raffinose, and 'polysaccharides', such as glycogen, dextran, and inulin. While precise assessment of inhibition is difficult because of the nature of the assay and the possibility of secondary effects such as varying permeability, there appears to be a trend toward greater inhibition by the more complex sugars, possibly related to the number of hydroxyl groups per molecule. The data also show that the inhibition of mitochondrial contraction is given not only by the true sugars, but also by polyols, including mannitol, sorbitol, and inositol.

Tests were also made of a variety of simpler alcohols and from these (see Table I) it was found that inhibition of the ATP-induced mitochondrial contraction apparently requires the alcohol to have more than 3 hydroxyl groups, since glycerol, ethylene glycol, diethylene glycol, and propylene glycol did not inhibit significantly even at concentrations as high as 2.0 *M*. Similarly methanol, ethanol, and propanol were not inhibitory at 0.3 *M*. The definite inhibitions noticed with *n*-butanol, isoamyl alcohol, and benzyl alcohol, however, could be caused by an action quite different from that of the sugars because these

TABLE I

Effect of Alcohols on Contraction of Thyroxine-swollen Rat Liver Mitochondria by ATP

Details as in Fig. 1. The compounds were tested at several concentrations.

Compound	Inhibition at 0.3 M	Concentration giving 50% inhibition M
Hexoses		
D-glucose	++++	<0.10
D-Fructose	++++	<0.10
D-Mannose	++++	0.10
L-Sorbose	++++	0.10
Pentoses		
Xylose	++++	0.15
Ribose	++	0.3
Disaccharides		
Sucrose	++++	0.05
Lactose	++++	0.05
Maltose	++++	0.05
Trisaccharide		
Raffinose	++++	0.15
Polysaccharides		
Liver Glycogen 6%	++++	1%
Dextran 6%	++++	1%
Inulin 6%	++++	1%
Alcohols		
Mannitol	++++	0.05
Sorbitol	++++	0.05
Myoinositol	++++	0.05
Ribitol	++	0.3
Glycerol	0	1.20
Ethylene glycol	0	2.0
Propylene glycol	0	1.0
Methanol	0	≥0.6
Ethanol	0	≥0.6
Propanol	0	>0.6
n-Butanol	+++	0.1
Isoamyl alcohol	+++	0.1
Benzyl alcohol	++	0.3
Diethylene glycol	0	>0.6

alcohols have a high degree of lipid-solubility and probably act by disrupting the lipoproteins of the mitochondrial membranes (*cf.* (13)).

While no attempt was made to assay a great number of compounds, it appears quite clear that ATP-induced contraction of swollen

mitochondria is inhibited by all polyhydroxylic compounds tested having more than 3 (adjacent) hydroxyl groups.

Comparison of Optical and Gravimetric Methods—Some additional tests were necessary to establish that the optical method, which measures light scattering indirectly (5), actually measured water uptake and extrusion by the mitochondria in sugar-containing media. Since light scattering by the mitochondria depends on, among other factors, the refractive index difference between medium and particles, it appeared possible that the relatively high concentrations of the sugars producing inhibition altered this refractive index difference sufficiently to obscure actual changes in mitochondrial volume, thus giving a spurious inhibition of the contraction. The inhibitory action of sucrose was therefore measured by the direct gravimetric method, in which the mitochondrial pellet is weighed following centrifugal recovery at zero time (before swelling), after swelling, and after addition of ATP. The gravimetric and optical data in Table II give good agreement and show that the pres-

TABLE II

Gravimetric and Optical Measurement of Water Extrusion in Sucrose Medium

Rat liver mitochondria were added to replicate tubes containing 10.0 ml. 0.02 M tris-HCl pH 7.4, 3×10^{-5} M L-thyroxine, and either 0.4 M sucrose or 0.125 M KCl as indicated. After maximum swelling had occurred, 0.005 M ATP + 0.005 M $MgCl_2$ + 0.4 mg. per ml. bovine serum albumin were added. Duplicate tubes were centrifuged at zero time, after swelling, and after contraction induced by ATP + Mg^{++} + SA, and wet weight of the mitochondrial pellet determined. Optical changes were measured at 520 mμ and are given for 1.0 mm. optical path. Temp., 25°C.

Medium	Time (min.)	State of mitochondria	D ₅₂₀	Wet Weight mitochondria mg.
1. KCl	0	Initial	0.229	155
	20	Swollen	0.140	386
	40	After ATP addition	0.209	134
2. Sucrose	0	Initial	0.260	115
	50	Swollen	0.184	266
	75	After ATP addition	0.176	240

ence of sucrose does in fact inhibit the ATP-induced decrease of mitochondrial weight. While the refractive index of the solutions listed in Table I contributes to the magnitude of the optical absorbance changes in such experiments the simple optical assay clearly appears to be a valid semiquantitative measure of the ability of the sugars to inhibit contraction. This conclusion is also supported by the finding that a variety of solutes other than polyols have no inhibitory action on mitochondrial contraction when tested at sufficiently high concentrations to make substantial contributions to the refractive index of the medium. The data in Table III show that sucrose and D-glucuronate are highly inhibitory, but that such solutes as urea, thiourea, acetamide, dioxane, acetone, and dimethylformamide do not significantly inhibit optically measured ATP-induced contraction at 0.3 *M*. To these must be added the non-inhibitory alcohols listed in Table I, such as glycerol and ethylene glycol.

TABLE III

Effect of Other Solutes on Mitochondrial Contraction

Details as in Table I and Fig. 1.

Compound	Inhibition at 0.3 <i>M</i>	Concentration giving 50% inhibition <i>M</i>
Sucrose (control)	++++	0.05
Acetamide	0	≥0.6
Urea	0	>1.2
Thiourea	+	>0.5
Dioxane	+	≥0.5
Acetone	+	≥0.3
Dimethylformamide	0	≥0.3
Sodium D-glucuronate	++++	>0.1

A second aspect of the assay which has been examined further is the ionic composition of the medium. Although the test media always contained 0.02 *M* tris-HCl buffer pH 7.4, no other ionic components were added. The normal medium giving maximum contraction response contains 0.125 *M* KCl. However, a preceding study (14) has shown that the ATP-induced contraction of swollen rat liver mitochondria is essentially independ-

ent of ionic composition or ionic strength within defined limits. It can proceed maximally when the cation is K⁺, Na⁺, Rb⁺, Li⁺, NH₄⁺ and tris⁺, or when Cl⁻ is replaced by I⁻, Br⁻, F⁻, acetate⁻, NO₃⁻, and SO₄²⁻, among others.

However since the assays of Table I lacked ions other than 0.02 *M* tris and Cl⁻, additional tests were made in which 0.125 *M* KCl was added to the sucrose-containing media. However the inhibitory action of sucrose was manifested in all media tested, and within the limitations of the semiquantitative nature of the assay, the inhibition by sucrose was not significantly affected by the presence of 0.125 *M* KCl.

Effect of Some Sugars on DNP-stimulated ATP-ase—Earlier work has shown that DNP-stimulated ATP-ase is virtually completely inhibited by 0.3 *M* sucrose in preparations of digitonin fragments of rat liver mitochondria (7), as is the ATP-*i*P³² exchange reaction (8). Since the digitonin particles do not change volume in response to changes in osmotic pressure of the medium (*cf.* (15)) it is probable that the inhibition of the ATP-ase is caused through some chemical interference in an intermediate enzymatic step rather than by a change in the geometry or volume of the particles produced by a change in osmolality.

The inhibition of DNP-stimulation of ATP-ase in such particles by a variety of sugars was measured under conditions which were optimal for the inhibition by sucrose. The pH of the medium is especially important, since the greatest inhibition by sucrose occurred at pH 7.5. Data in Table IV show that the DNP-stimulated ATP-ase is inhibited not only by sucrose, but also by glucose, fructose, mannitol, inositol, and xylose, but not by glycerol. These findings thus fully parallel the data on mitochondrial contraction. However it may be noted that the sugars vary more widely in their inhibitory potency toward ATP-ase than they do in the inhibition of mitochondrial contraction. It is seen for example that xylose is only slightly inhibitory to the ATP-ase, compared to

TABLE IV

Effect of polyols on DNP-stimulated ATP-ase

Test system contained 0.006 *M* ATP, 5×10^{-5} *M* 2, 4-dinitrophenol, 0.01 *M* tris-HCl buffer pH 7.5, digitonin fragments (171 μ g. N) and polyols shown in total volume of 1.0 ml. Incubated 15 min. at 20°C.

Compound	<i>M</i>	iP formed m μ moles	Inhibition percent
None (control)	—	1,230	—
"	—	1,270	—
Sucrose	0.1	940	25
"	0.3	340	73
"	0.5	47	96
Glucose	0.1	1,190	5
"	0.3	910	27
"	0.5	420	66
Fructose	0.1	1,180	6
"	0.3	1,020	18
"	0.5	642	48
Mannitol	0.1	940	25
"	0.3	220	83
"	0.5	210	83
Inositol	0.3	650	48
"	0.5	120	90
Xylose	0.3	1,240	0
"	0.5	980	22
Glycerol	0.3	1,270	0
"	0.5	1,200	0
"	0.9	1,240	0

sucrose, whereas it is relatively much more inhibitory to mitochondrial contraction. On the whole, however, the parallelism between the two types of assay system is rather uniform, and supports the view that the processes share a common reaction which is sensitive to sucrose and other polyhydroxylic compounds.

Inhibition of Respiration and Phosphorylation—

Sucrose also inhibits respiration and decreases the P: O ratio in digitonin particles (10) as well as in intact mitochondria (16). Tests in Table V show the inhibition of respiration in rat liver mitochondrial particles prepared with digitonin by sugars and alcohols. In general, those sugars and alcohols inhibiting mitochondrial contraction and ATP-ase also

were found to inhibit oxidation of β -hydroxybutyrate by oxygen, as well as the coupled phosphorylation. On the other hand, glycerol was ineffective. Again it is to be noted that xylose is less active than the higher sugars. These findings are also consistent with the view that a common enzymatic reaction inhibited by sucrose and other sugars is shared by the processes of oxidative phosphorylation, DNP-stimulation of ATP-ase, and the contraction of swollen mitochondria by ATP.

TABLE V

Inhibition of Respiration and Phosphorylation by Representative Polyols

The test system contained 0.01 *M* β -hydroxybutyrate, 0.0024 *M* ADP, 0.03 *M* phosphate pH 6.5, and digitonin fragments (134 μ g. N) in a 1.0 ml. system. Polyols added in concentrations shown. Incubated 15 minutes at 20°C.

Compound	<i>M</i>	Acetoacetate formed m μ moles	P : O
None	—	286	1.48
"	—	255	1.52
Sucrose	0.1	215	1.55
"	0.3	121	0.93
"	0.5	83	0.32
Inositol	0.1	235	1.61
"	0.3	212	1.21
"	0.5	124	1.13
Xylose	0.1	248	1.53
"	0.3	215	1.64
"	0.5	190	1.32
"	0.7	140	1.21
Glycerol	0.1	242	1.56
"	0.3	255	1.50
"	0.5	235	1.58
"	1.0	242	1.49

DISCUSSION

The findings reported here clearly show that sucrose and a variety of other polyhydroxylic compounds inhibit contraction of thyroxine-swollen mitochondria by ATP. While the conditions of the assay do not permit precise quantitative evaluation, it is

quite clear that the inhibition is given only by compounds having more than 3 hydroxyl groups per molecule and not by simple alcohols or a variety of other water soluble non-ionic organic compounds. Among the active polyols, the severity of inhibition increases with the number of hydroxyl groups per molecule. No attempt was made however to examine all aspects of molecular structure and functional group reactivity contributing to this inhibition.

A survey of the effect of these polyols on DNP-stimulated ATP-ase and the rates of respiration and phosphorylation in digitonin particles, which do not undergo osmotic swelling and shrinking, showed that the contraction-inhibiting compounds also inhibit these partial reactions of oxidative phosphorylation, giving further support to the hypothesis expressed earlier that mitochondrial swelling and contraction and the mechanism of energy-coupling, both functions of the membrane(s), share a common intermediate enzymatic reaction which is inhibited by sucrose and, as this study has shown, by other polyhydroxylic compounds also. Other functional relationships between the swelling and contraction cycle and the mechanism of oxidative phosphorylation have been described in full elsewhere (4, 5)

Polyhydroxylic compounds inhibitory to the contraction, such as sucrose, glucose, mannitol, are not known to have inhibitory action on enzymes in general and their chemical nature does not suggest they should inhibit or destroy the various functional groups currently believed to be involved in the action of enzymes. However, there is a possible chemical explanation for the action of sucrose which is well-founded in current knowledge of enzyme action. A number of hydrolytic enzymes have been described which show "transferase" activity. For example, work of Morton and others (*cf.* (17, 18)) has shown that when sucrose or other alcohols are present in high concentrations they can accept phosphate from substrates such as α -glycerophosphate in the presence of alkaline phosphatase. Such "transferase" activity has

been depicted as occurring through competition between the alcohol or sugar (ROH) with HOH. "Transferase" activity is also seen among carbohydrate-hydrolyzing enzymes and peptidases in the presence of appropriate acceptors. Usually relatively high concentrations of the acceptor are required to compete successfully with the large mole-fraction of water in aqueous systems.

It appears possible that such a mechanism may be involved in the inhibitory action of sucrose and other polyols on an intermediate reaction of oxidative phosphorylation. The sucrose may either compete with water molecules or, more likely, with an active site on an intermediate enzyme containing a hydroxyl group(s) functional in a group-transfer reaction. Using the following reaction scheme for oxidative phosphorylation possible actions of sucrose as an artificial acceptor in a group-transfer reaction may be shown:

- (1) $\text{Carrier} \sim \text{X} + i\text{P} \longrightarrow \text{Carrier} + \text{P} \sim \text{X}$
- (2) $\text{P} \sim \text{X} + \text{E} \longrightarrow \text{P} \sim \text{E} + \text{X}$
- (3) $\text{P} \sim \text{E} + \text{ADP} \longrightarrow \text{ATP} + \text{E}$

where X and E are group-transferring enzymes. One possible action of sucrose based on a "transferase" reaction may be indicated by reactions (4) and (5)

- (4) $\text{P} \sim \text{X} + \text{Sucrose} \longrightarrow \text{Sucrose-phosphate} + \text{X}$
- (5) $\text{P} \sim \text{E} + \text{Sucrose} \longrightarrow \text{Sucrose-phosphate} + \text{E}$

in which sucrose serves as phosphate acceptor instead of the normal acceptors E and ADP respectively.

Experiments have been carried out to test this hypothesis. Sucrose has been added to systems in which oxidative phosphorylation was taking place in the presence of inorganic phosphate labeled with P^{32} of very high specific activity. The reaction media were fixed with perchloric acid and the filtrates chromatographed on Dowex-1 columns and a search made for the appearance of P^{32} -labeled phosphate ester(s) of sucrose. Within the sensitivity limitations of such experiments no unidentified peaks of radioactivity could be found. From this experiment and other approaches, interaction of sucrose with an intermediate phosphate ester to produce phosphorylated sucrose seems unlikely.

There is however a possibility that sucrose participates in a "transferase" reaction which does not involve phosphate specifically, but some other functional group. For example, the hypothetical high energy carrier complex, $\text{Carrier} \sim \text{X}$, may undergo a "transferase" reaction with sucrose rather than with the inorganic phosphate, to form either $\text{Carrier} \sim \text{sucrose}$ or $\text{sucrose} \sim \text{X}$. Sucrose in high concentrations may thus keep a large fraction of the total amount of Carrier or X in such a presumably inactive form, leading to inhibition of the overall cycle. Since the inhibitory action of sucrose is reversible, it is necessary to postulate that such sucrose-intermediate esters undergo decomposition.

The swelling of mitochondria is also inhibited by sucrose and other sugars, as shown earlier. However, this phenomenon is less sensitive to sucrose and the other agents (1, 2) than is contraction. In the assays described here, a large excess of the swelling agent (thyroxine) was employed, the action of which was not ordinarily overcome by the concentrations of polyols tested.

It has been postulated (1-5) that this action of sucrose is the basis for the efficacy of sucrose solutions in preserving mitochondrial morphology during isolation, *i.e.* a "fixation" of morphology by inhibition of intermediate enzymes involved in swelling and contraction. On the basis of the results reported in this paper, the efficacy of such widely used polyhydroxylic solutes as mannitol (19), raffinose (20), and dextran (20) must have a similar basis.

It is very curious that there is a striking inverse relationship between the relative inhibitory potency of the polyhydroxylic compounds, tested against ATP-induced contraction of mitochondria, and the relative rate of penetration through the membrane of protoplasts of *Staph. aureus* and *M. lysodeikticus*, as revealed by the measurements of Mitchell and Moyle (21). Thus sucrose is a very slow penetrant but a strong inhibitor, whereas glycerol is a fast penetrant and does not inhibit contraction. As an alternative to the hypothesis proposed above, it appears

possible that the non-penetrating sugars may be bound or adsorbed to the mitochondrial membrane, at or near sites normally permitting entry of smaller polar molecules such as glycerol or ethylene glycol. Such adsorption of sucrose may prevent access of ATP to the specific sites in the membrane involved in the contractile mechanism.

The inhibition of mitochondrial contraction by sucrose described in this and earlier papers serves to differentiate the large amplitude mitochondrial swelling and contraction observed *in vitro*, in which the mitochondria may increase and decrease in volume over a two- or three-fold range, from the rapid, relatively minute volume changes observed by Packer in fresh, "tightly-coupled" mitochondria when changes in respiratory state are induced (22). The latter type of changes, both swelling and contraction, were found by Packer not to be affected by concentrations of sucrose as high as 0.80M.

SUMMARY

Sucrose in concentrations of 0.3M inhibits ATP-induced contraction of thyroxine-swollen rat liver mitochondria as measured optically or gravimetrically. This effect is not due to an impurity but is given by a wide variety of other polyhydroxylic compounds, including glycogen, dextran, inulin, raffinose, maltose, lactose, mannitol, sorbitol, inositol, glucose, fructose, mannose, sorbose, ribose, and xylose. Half-maximum inhibition is given by between 0.05 and 0.10M sugar. On the other hand, simpler alcohols such as glycerol, ethylene glycol, ethanol, methanol, or solutes such as acetone, urea, dioxane, and acetamide do not interfere in ATP-induced contraction at concentrations up to 1.0M. The polyols which inhibit mitochondrial contraction also inhibit dinitrophenol-stimulated ATP-ase of mitochondrial membrane fragments with parallel efficacy, as well as coupled respiration in such particles. It is concluded that the polyols inhibit an intermediate reaction of oxidative phosphorylation shared by the ATP-driven contractile mechanism of the membrane, possibly by serving as artificial ac-

ceptors in an essential enzymatic group transfer reaction. An alternate explanation suggests that the inhibitory polyols block access of ATP to membrane sites concerned in contraction.

REFERENCES

- (1) Lehninger, A. L., Ray, B. L., and Schneider, M., *J. Biophys. Biochem. Cytol.*, **5**, 97 (1959)
- (2) Lehninger, A. L., *J. Biol. Chem.*, **234**, 2187 (1959)
- (3) Lehninger, A. L., *J. Biol. Chem.*, **234**, 2465 (1959)
- (4) Lehninger, A. L., *Ann. N. Y. Acad. Sci.*, **86**, 484 (1960)
- (5) Lehninger, A. L., *Physiol. Rev.*, in press.
- (6) Tapley, D. F., *J. Biol. Chem.*, **222**, 325 (1956)
- (7) Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, **224**, 547 (1957)
- (8) Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, **224**, 561 (1957)
- (9) Lehninger, A. L., Wadkins, C. L., Cooper, C., Devlin, T. M., and Gamble, J. L. Jr., *Science*, **128**, 450 (1958)
- (10) Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, **219**, 489 (1956)
- (11) Devlin, T. M., and Lehninger, A. L., *J. Biol. Chem.*, **233**, 1586 (1958)
- (12) Dickens, F., and Salmony, D., *Biochem. J.*, **64**, 645 (1956)
- (13) Morton, R., *Nature*, **166**, 4073 (1950)
- (14) Lehninger, A. L., *Biochim. et Biophys. Acta*, in press.
- (15) Packer, L., and Tappel, A. L., *J. Biol. Chem.*, **235**, 525 (1960)
- (16) Johnson, D., and Lardy, H. A., *Nature*, **181**, 701 (1958)
- (17) Morton, R. K., *Nature*, **172**, 65 (1953)
- (18) Morton, R. K., *Discussions Faraday Society*, **20**, 149 (1955)
- (19) Raaflaub, J., *Helv. Physiol. Pharmacol. Acta*, **11**, 142, 157 (1953)
- (20) Birbeck, M. S. C., and Reid, E., *J. Biophys. Biochem. Cytol.*, **2**, 609 (1956)
- (21) Mitchell, P., and Moyle, J., *J. Gen. Microbiol.*, **6**, 150 (1956)
- (22) Packer, L., *J. Biol. Chem.*, **235**, 242 (1960)

Radioactivation Analysis of Strontium in Rat Bone Ash

By YOSHIHIRO MATSUMURA and REIKO FUJINO

(From the Department of Biochemistry, Tokyo Women's Medical College, Tokyo)

(Received for publication, February 28, 1961)

The concentration of strontium normally present in most of biological materials is so low as to be beyond the reach of conventional analytical methods. Consequently the reported values are too discordant to be of much value. Recently Sowden and Stitch (1), investigating trace elements in human tissues, reported on the application of neutron activation analysis on the determination of bone strontium. The turnover of strontium can be elucidated more fully by the determination of stable strontium existing in tissues than by the simple tracer tests.

Since 1958 the use of Japan Research Reactor I (JRR-1) was open to general scientific studies, which made possible the neutron activation analysis of biological trace elements (2).

The purpose of the study is to attempt the use of JRR-1 for neutron activation analysis of strontium as a basis of turnover study on rat bone under various nutritional conditions.

EXPERIMENTALS

Reagents—Carriers.

Strontium carrier. A standard solution of strontium chloride was used. The concentration is assayed as strontium carbonate gravimetrically.

Barium carrier. Barium nitrate (1.903 g.) was dissolved in distilled water to make up 100 ml. 1 ml. of the solution contains 10 mg. of barium.

Iron carrier. Ferric nitrate (7.35 g.) was dissolved in distilled water to make up 100 ml. 1 ml. of the solution contains 10 mg. of iron.

Strontium standard. Strontium nitrate ($\text{Sr}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) was diluted with calcium carbonate to 1:1000 in an agate mortar. 323.6 mg. of the diluted mixture (containing 100 μg . of Sr) was weighed and mixed further well with ca. 2 g. of calcium carbonate.

Other reagents. Fuming nitric acid, specific

gravity 1.53; 17 *N* NH_4OH , 28% aqueous solution of ammonia; Acetate buffer, 3 *M*, pH 4.7, equal volumes of 3 *M* acetic acid and 3 *M* sodium acetate are mixed; 1.4 *M* Potassium chromate; Hydrogen peroxide, 30%; Methyl alcohol.

Animals—Five albino rats, between two and three months of age and 2000 to 3000 g. in weight, were maintained in iron metabolic cages and were fed with Oriental chow MF for at least three weeks. Distilled water was given freely throughout the whole period. Femurs were removed carefully from soft tissues, soaked overnight in 1 *M* KOH, rinsed with distilled water thoroughly. The femurs were combined and placed in a silica crucible, dried for one hour at 100°C, then ashed at 500°C for 7 hours in an electric muffle furnace. The ash was ground to a fine powder in an agate mortar.

Activation of Samples and Standards—Two standard samples, each consisting of a weighed mixture of strontium nitrate and calcium carbonate (containing 100 μg . of strontium) and six samples of bone ash, each wrapped in polyethylene sheets, were introduced into a small polyethylene containers as to be irradiated simultaneously.

The samples were irradiated in air-driven tubes of JRR-1 for two hours. For a week or 4 weeks, they were placed in one of the experimental holes. The containers were subjected to a neutron flux of 10^{11} neutrons $\text{cm}^{-2} \text{sec}^{-1}$.

After two hours of irradiation, the surface activity of the polyethylene container was at the level of 1500 mr/hour. It decreased to 700 mr/hour after 10 minutes. The specimens were left for 30 minutes before handling to allow the decay of intense radioactivity from isotopes of a short half-life.

After one or four weeks of irradiation, the surface activity was 50 mr/hour on the fourth day after irradiation.

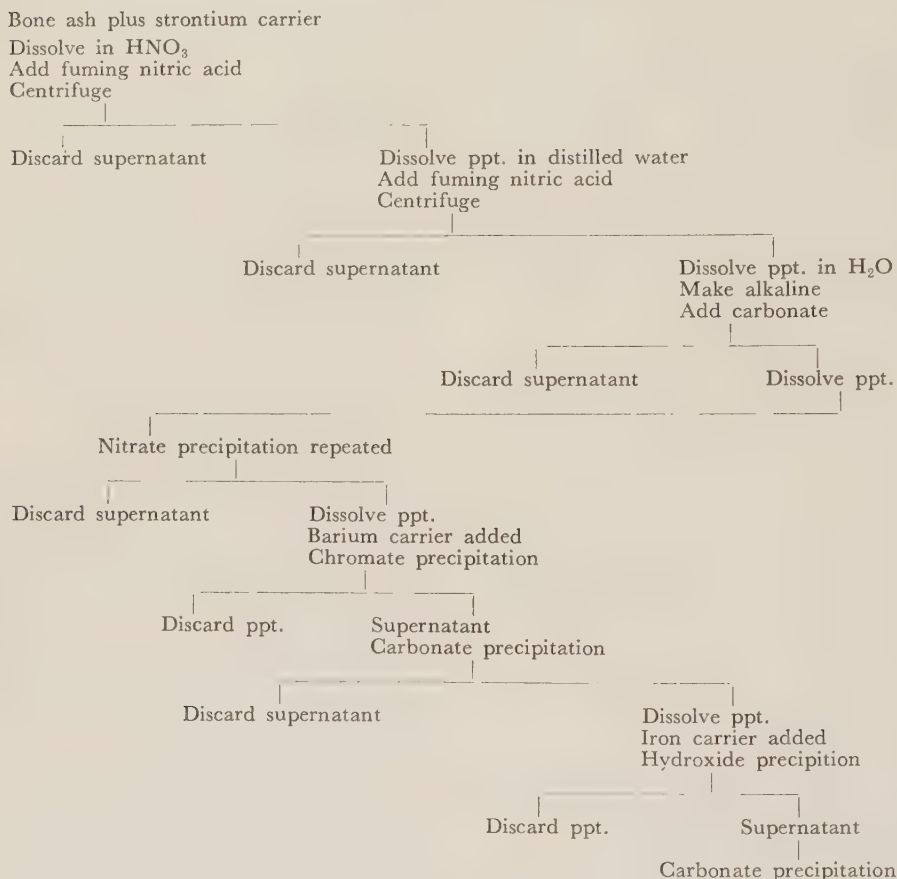
Radiochemical Purification (3)—All chemical manipulations were carried out behind a lead shielding of 10 cm. thickness. Discarded radioactive solution were stored in polyethylene bottles placed behind a lead shielding of 10 cm. thickness during the analytical procedure.

After activation of the bone ash and strontium standard, each of them was weighed into numbered 50 ml. glass stoppered centrifuge tubes. Approximately 2.0 ml. (containing 64 mg. Sr) of the solution of strontium carrier, and 6.0 ml. of distilled water were added to each of them, and then 10 ml. of fuming nitric acid were carefully added to dissolve the ash.

tube, washing twice with 2 ml. of distilled water. The combined washings were neutralized with 17 *N* NH_4OH , methyl red being added as an indicator, made distinctly alkaline with ammonia. 0.5 g. of ammonium carbonate was added to precipitate strontium carbonate. After heating in a boiling water bath for 5 minutes and centrifuging, the supernatant

SCHEME I

Scheme of radiochemical purification.



Strontium were precipitated by the addition of further 10 ml. of fuming nitric acid, cooled in an ice bath for more than 10 minutes, and then separated by centrifugation ($1,000\times g$, 10 minutes) the supernatant liquid being discarded. The insoluble nitrates were dissolved in 5.0 ml. of distilled water, reprecipitated by the addition of 10 ml. of fuming nitric acid and the supernatant was discarded as before.

The precipitate was redissolved in 2 ml. of distilled water and transferred into a 15 ml. centrifuge

was discarded. The precipitate was dissolved in minimum quantity of distilled water, strontium was reprecipitated as nitrate. Nitrate precipitation was repeated once more. The precipitate was dissolved in 2 ml. of distilled water with the addition of 1 ml. of the solution of barium carrier, neutralized with 2 *N* NH_4OH under the indication of methyl red, and then 2 ml. of acetate buffer was added. After heating in a boiling water bath for 5 minutes, 0.5 ml. of 1.5 *N* potassium chromate was added. After further

heating for 5 minutes in a boiling water bath and cooling to room temperature, centrifuged for 10 minutes at $1000\times g$ and the supernatant was transferred into another 15 ml. centrifuge tube. The supernatant was made alkaline with the addition of 17 *N* NH_4OH and 0.5 g. of ammonium carbonate was added to precipitate strontium as carbonate. After heating in a boiling water bath for 5 minutes and cooling to room temperature, it was centrifuged ($1000\times g$, 10 minutes) and the supernatant was discarded. The precipitate was dissolved in 2 ml. of 2 *N* HNO_3 , and one drop of hydrogen peroxide was added to reduce chromate. After addition of 1 ml. of iron carrier, and heating in a boiling water bath to remove carbon dioxide, it was made alkaline with the addition of 17 *N* NH_4OH . Heating in a boiling water bath for 5 minutes, iron was precipitated completely as ferric hydroxide. After centrifugation ($1000\times g$, 10 minutes), the supernatant was transferred into polyethylene tubes and the radioactivity was measured in a well-type scintillation counter.

After the assay of radioactivity, the solution of strontium was quantitatively transferred into a centrifuge tube for the estimation of the chemical yield. Strontium was precipitated as carbonate with excess of ammonium carbonate. The precipitate was centrifuged, washed once with dilute aqueous NH_3 solution, and transferred into weighed steel planchet with the aid of methyl alcohol. The sample was dried under an infra-red lamp to a constant weight. The precipitate was equilibrated to atmospheric CO_2 for two hours before weighing.

The radioactivity was also measured by conventional Geiger-Müller counter or Lauritzen type electrometer in the form of strontium carbonate.

RESULTS

The gamma ray spectrograms of radiochemically purified specimen and of the standard are shown in Fig. 1. Authentic Hg-203 was used to calibrate the level of gamma ray energy, assuming the main peak of Hg-203 was situated at 0.279 MEV (4). Strontium standard showed three distinct peaks. Most of the activities were due to the production of Sr-87m. To a lesser extent, Sr-85m and Sr-85 were also detected. From the area of main peak, content of strontium in bone ash was calculated as shown in Table V.

The results of the experiment to know

to what extent the removal of coexisting calcium in bone ash is effected by the radiochemical purification are shown in Table I and II.

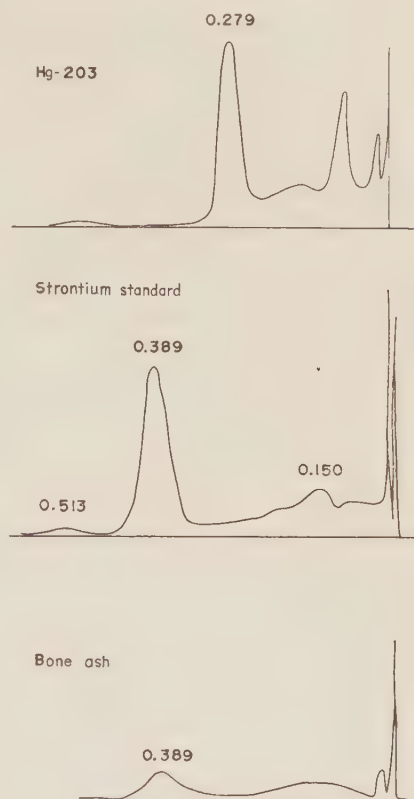


FIG. 1. Gamma ray spectrogram obtained by 256-channel pulse height analyzer.

TABLE I
Removal of Calcium, assayed with
Ca-45 as a Tracer

Composition of Samples Ca CO_3 (g.)		Ca^{45} (μc)	Sr (mg.)
a)	2	100	0
b)	2	100	64.08
c)	—	100	64.08
d)	—	—	0

Table III and IV are obtained as results of one week or longer irradiations.

TABLE II
Results Given in Percentage of Removed Calcium

Stage of procedure	Assayed	Samples			
		a)	b)	c)	d)
First nitrate precipitation	Supernatant	97.0	88.5	88.5	(-)
Second nitrate precipitation	"	2.9	10.4	10.4	(-)
Carbonate precipitation	"	(-)	(-)	(-)	(-)
Third nitrate precipitation	"	0.1	1.0	1.0	(-)
Fourth nitrate precipitation	"	(-)	0.4	0.1	(-)
Barium chromate scavenging	"	(-)	(-)	(-)	(-)

(-): No detectable radioactivity of Ca^{45} .

TABLE III
Activities Induced in Bone Ash and Strontium Standard after
Two Hours Irradiation

Sample	Weight (g.)	SrCO_3 recovered (mg.)	Radioactivity	
			counts per minutes	division per minute
Standard 100 $\mu\text{g.}$	0.9903	43.6	28.6	5.68
Standard 100 $\mu\text{g.}$	0.3146	71.3	27.4	4.92
Standard 200 $\mu\text{g.}$	1.2508	92.5	91.4	8.00
Standard 200 $\mu\text{g.}$	0.6396	89.7	67.9	8.30
Bone ash	0.9951	77.1	32.5	6.54
Bone ash	0.9832	46.4	20.2	6.07
Bone ash	0.9888	87.5	38.7	6.64
Bone ash	0.9934	87.4	33.9	5.39

TABLE IV
Activities Induced in Bone Ash Strontium Standard after
Four Weeks Irradiation

Sample	Weight (g.)	SrCO_3 recovered (mg.)	Radioactivity	
			counts per minutes	division per minute
Standard 100 $\mu\text{g.}$	0.9839	66.6	50.8	9.82
Standard 100 $\mu\text{g.}$	0.3054	66.3	88.4	8.63
Standard 200 $\mu\text{g.}$	1.0644	76.5	253.9	16.03
Standard 200 $\mu\text{g.}$	0.6296	69.1	209.3	13.21
Bone ash	1.0685	30.0	99.7	9.25
Bone ash	0.9891	32.6	47.3	6.25
Bone ash	0.8352	32.1	86.5	11.40
Bone ash	1.0281	32.5	78.5	9.83

TABLE V
Calculation of Strontium Content of Bone Ash

Irradiation time	Instrument used	Strontium in bone ash
		$\mu\text{g./g.}$
Two hours	256-Channel pulse height analyser	120
One week	Geiger-Müller counter	108.5
	Lauritzen electrometer	137.6
Four weeks	Geiger-Müller counter	174
	Lauritzen electrometer	111.4

DISCUSSION

Strontium has four stable nuclides. Activation by thermal neutron flux will produce several kinds of radioactive nuclides. They

ray spectrogram and also from their half-life and Feather diagram.

SUMMARY

1. The application of neutron activation analysis to quantitation of stable strontium in bone ash is discussed. The Japan Research Reactor I was utilized as a source of neutron flux of 10^{11} neutrons/cm²/sec. The results obtained by the irradiation for two hours and one week or longer were compared.

2. The radiochemical purification gave the recovery of strontium as about 76–86 per cent. Calcium or other coexisting elements were completely removed by the procedure.

3. Strontium content of rat bone ash, which was fed for three weeks or more with Oriental chow MF, were within the range of 120–180 microgram per gram.

TABLE VI
Thermal Neutron Activation of Strontium

	Sr-84	Sr-86	Sr-87	Sr-88
Stable nuclides				
Natural abundance	0.56	9.86	9.02	82.56
Thermal neutron cross section (barn)	1	1.3?		0.005
	Sr-85 m	Sr-85	Sr-87 m	Sr-89
Activated nuclides				
Half-life	70 min.	65 day	170 min.	54 day
Radiation and its energy (MEV)	IT 0.007 γ 0.233 K 0.150	γ 0.513	γ 0.389	β 1.48

were summarized in Table VI. These nuclides might be produced from rubidium and yttrium, which are scarcely contained in living materials. The irradiation for two hours produces mainly Sr-87m and Sr-85m. One week or longer irradiation of strontium produces all the radioactive nuclides listed in the table. During cooling for seven days the activity due to Sr-85m and Sr-87m decayed off and there remained only that of Sr-85 and Sr-89.

Various activities induced from all the elements other than strontium in bone ash were completely removed by the purification procedure. This was known from the gamma

The authors wish to express their thanks to Prof. H. Yoshikawa for his helpful discussion and encouragement during the course of this work. They wish also to extend their thanks to Dr. T. Chitani for his concern in distributing a grant from the Ministry of Education.

REFERENCES

- (1) Sowden, E. M., and Stitch, S. R., *Biochem. J.*, **67**, 104 (1957)
- (2) Matsumura, Y., *Saishin Igaku*, **15**, 913 (1960)
- (3) Bryant, F. J., Chamberlain, A. C., Morgan, A., and Spicer, G. S., AERE HP/R. 2526 (1956)
- (4) Kinsman, S., "Radiological Health Handbook," U. S. Department of Health, Education, and Welfare. Bureau of State Services, Cincinnati, Ohio.

The Quantitative Determination of Arginine

By SHIGERU AKAMATSU and TAKEO WATANABE

(From the Department of Biochemistry, School of Medicine,
Chiba University, Chiba)

(Received for publication, February 28, 1961)

Red colorization of alkaline solution of arginine by addition of α -naphthol and hypochlorite is known as Sakaguchi's reaction (1). Hypobromite and oxine (8-hydroxyquinoline) were preferred by Weber (2) and Sakaguchi (3) to hypochlorite and α -naphthol, respectively. Based on this principle several procedures for colorimetric determination of arginine have been reported. In most of them successive addition of urea was carried out to eliminate any hypobromite exceeding an amount necessary to dye formation, so that the dye once formed may not be decomposed by further action of hypobromite. However, because of the rapid development and successive fading of the color, the time interval between hypobromite and urea additions was strictly fixed and should not deviate from it even by a few seconds. It would be convenient if there were any substance which would in case be added beforehand to the test solution, not interfere with the dye formation, yet be able to inactivate the excessive hypobromite. After testing several compounds, sulfosalicylic acid was found to fit for the purpose and the colorimetric determination of arginine has become significantly simple to carry out, as is described in the following.* In the present method oxine recommended by Sakaguchi (3) as another coupling reagent is used instead of α -naphthol.

EXPERIMENTAL AND RESULT

Reagents—1) Sulfosalicylic acid-oxine solu-

tion. 50 ml. of 5% (about 0.2 *M*) sulfosalicylic acid and 50 ml. of 0.01 *M* glycine are mixed and therein 0.05 g. oxine is dissolved.

2) 2.5% NaOH solution.

3) Hypobromite solution. 1 g. bromine is dissolved in 100 ml. of 5% NaOH. Useful at least for 2 months, when preserved cold in a brown bottle.

4) Standard *M*/4000 solution of arginine for calibration. 0.01 *M* arginine monohydrochloride solution, preserved under toluene layer in an ice box, is diluted at every usage. 1 ml. of it contains 43.5 μ g. of arginine.

Procedure—To 2 ml. of a test solution is added 2 ml. of sulfosalicylic acid-oxine solution and filtered. 2 ml. of the deproteinized filtrate is pipetted into a test tube, mixed with 1 ml. of 2.5% NaOH and refrigerated in ice water for about 15 minutes. Then 1 ml. of hypobromite solution is added by a measuring pipette equipped with a gummi cap and the mixture wherein momentarily the maximal dye formation takes place is diluted to the 10 ml. mark with water of room temperature not necessarily at a fixed lapse of time, but freely within 10 minutes. A longer leaving is undesirable since later than 15 minutes a slight fading of color may occur. After dilution with water, colorimetry is carried out immediately, but some minutes delay does not affect the extinction. When 1 ml. of the standard *M*/4000 arginine solution and 1 ml. of sulfosalicylic acid-oxine solution were mixed and treated as above mentioned, the extinction coefficient measured with Pulfrich-photometer, filter S 50 being used, was 0.71. The results are reproducible. In the absence of arginine, the dye formation is negligible and Beer's Law is obeyed in the range of

* The details were communicated in Japanese in the Journal of Chiba Medical Society, **34**, 86 (1958) by T. Watanabe.

TABLE I

Effect of Glycine on the Dye Formation

Conc. of glycine added	M/10	M/50	M/100	M/200	M/400	M/800
Optical density	0.21	0.65	0.71	0.72	0.69	0.68

1 ml. of M/4000 arginine+1 ml. of 0.05% oxine+1 ml. of 2.5% sulfosalicylic acid+1 ml. of glycine solution of the concentrations listed. To this mixture is added 1 ml. of 2.5% NaOH. Further treatment as described in the text.

TABLE II

Inhibition of Color Development with Some Nitrogenous Compounds

O. D. in the presence of 1 ml. of the solution	M/50	M/100	M/200	M/400	M/800	M/1600	M/3200	M/4000
Urea		0.63	0.71	0.70	0.71			
Ammonium sulfate			0.65	0.71	0.70	0.71		
Glycine	0.48	0.71	0.71	0.70				
Alanine	0.62	0.71	0.70	0.71				
Leucine			0.62	0.63	0.71	0.70		
Tyrosine			0.63	0.70	0.69	0.70		
Tryptophane				0.64	0.70	0.71	0.71	
Histidine						0.63	0.68	0.71
Creatine			0.58	0.61	0.71	0.70		
Creatinine			0.65	0.70	0.71	0.70		
4-Methyl uracil		0.54	0.59	0.71	0.71	0.71		

Thymine M/5 (0.10)¹⁾ M/25 (0.34)¹⁾ M/125 (0.47)¹⁾

1 ml. of M/4000 arginine+1 ml. of the solution listed above+1 ml. of sulfosalicylic acid-oxine solution are treated as described in the procedure.

1) Optical density in the case of addition of thymine of the corresponding concentrations.

TABLE III

Optical Density of the Dye Solutions Produced from Guanidino Compounds

Compound	Optical density
Arginine	0.71
Arginic acid	0.71
Monoacetyl arginine	0.72
Agmatine	0.64
Hydroxy butyl guanidine	0.54
β -Guanidino propionic acid	0.60
Glycocyanine	0.47

1 ml. of M/4000 solution of the compounds listed+1 ml. of sulfosalicylic acid-oxine solution are treated as above.

M/2000 to M/32000 concentration of arginine solutions, when 1 ml. of them is employed to the test.

Remarks—Sulfosalicylic acid-oxine solution used in this method contains glycine at M/200 concentration. The presence of 1 ml. M/100 to M/200 glycine seems to increase the dye formation, as Table I indicates. The color intensifying effect of glycine is possibly due to a protection of arginine from the oxidative deamination and decarboxylation, since a shortening of the carbon chains of guanidino compounds causes the decrease of chromogenic activity, as will be later stated.

An excessive amount of glycine is not desirable since it rather deprives of hypobromite necessary for the maximal dye forma-

tion. The effects of various substances were also tested when they were present together with 1 ml. of *M*/200 glycine already contained in the sulfosalicylic acid-oxine solution. The results are indicated in Table II.

Histidine in an amount exceeding 1 ml. of *M*/4000 inhibits the color development, as in the case of the report of Macpherson (4). Applying also 1 ml. of various concentrations of other substances, the limits of their amounts, above which any inhibiting effect became observable were found to be below the ranges of their physiological occurrence in a test solution.

Sakaguchi's reaction is specific for monosubstituted guanidino compounds. When the length of carbon chains of the substituting groups is less than six, the color to be developed becomes less intensive.

DISCUSSION

The present method for arginine determination is certainly simpler than those previously reported by other authors. Urea addition for decomposition of excessive hypobromite was introduced by Weber (2). According to this procedure urea should be mixed in 4 to 6 seconds after hypobromite has been added. In the modifications of Weber's method by Jorpes and Thorén (5), Fischer and Wilhelmi (6) and Thomas Ingalls and Luck (7) the time preceding urea addition was somewhat lengthened but should be exactly 15 seconds. These manipulations are not convenient in practice, especially when not a few samples should be tested by one person. In Macpherson's method (4) the alkalinized solution of arginine is mixed beforehand with urea, followed by hypobromite addition and this urea-hypobromite treatment is once more repeated. However, it is said that Beer's Law is not obeyed. Van Pilsum *et al.* (8) recommended a beforehand addition of thymine to increase the extinction, however an employment of such an expensive reagent as thymine seems to be not practical and moreover the method does not provide any higher extinction than that obtainable with the present method. In

the method of Ceriotti and Spandrio (9) urea addition was omitted and alternatively the dye formed was extracted with butanol exactly after 10 seconds; the rigid adherence to time is not practical.

In the new method presented in this paper sulfosalicylic acid has been beforehand added, which eliminates hypobromite exceeding any amount necessary to the dye formation from arginine and oxine, so that the color produced can safely be left at least for 10 minutes without any further treatment for protection against fading. Of course, the optical density is dependent on the amounts of sodium hydroxide and hypobromite solutions to be added. Their appropriate amounts to give the highest extinction have been chosen and used. The concentration of oxine, recommended by Sakaguchi (3) as an coupling reagent, is lowered to make the extinction to nil when arginine is absent in the test solution.

The method can be applied to the measurement of arginase activity, estimating the remaining amount of arginine, whereby sulfosalicylic acid-oxine solution serves as a deproteinizing agent without a loss of oxine in the filtrate. Arginine added to a suspension of animal tissue, for instance, of muscle, could be quantitatively found in the filtrate obtained by addition of sulfosalicylic acid-oxine solution. The reagent can be employed to detect the spots on the paper chromatogram of guanidino compounds. The present method was used to measure arginine in serum and the results have been reported elsewhere by Ishikawa (10) from this institute. It would be mentioned here that serum contains in addition to arginine and glycocyamine some other substances positively reactive to Sakaguchi's reaction, which probably belong to polypeptides, since they are not precipitated by sulfosalicylic acid but by trichloroacetic acid and tends to increase in amount when the serum is left standing at 37°C. Arginine in serum could be determined therefore as follows. The trichloroacetic acid filtrate is neutralized and adjusted to pH 9. Before and after one hour action

of arginase (dialyzed liver autolysate) the modified Sakaguchi's method is carried out as mentioned in this paper and the arginine content is calculated from the difference of optical densities observed. The optical density of the sample treated after arginase action gives the amount of glycoamine, since no other guanidino compounds have been detected by paperchromatography carried out with the neutralized and desalted concentrate of filtrate obtained by deproteinizing serum with trichloroacetic acid.

SUMMARY

Sakaguchi's method of colorimetric arginine determination was modified. Hypobromite exceeding the amount necessary to dye formation was eliminated by a beforehand addition of sulfosalicylic acid to the test solution. The procedure has become far simpler than by other methods previously devised, since any treatment with urea or other compounds after hypobromite addition is unnecessary. The results are reproducible and Beer's Law persists within the range of 5.45 to 87 μ g. arginine. Sulfosalicylic acid can serve by itself as a deproteinizing agent in the case of arginase studies with dialyzed tissue

autolysate and arginine added to tissue suspension can be quantitatively recovered. Optical densities of the dyes produced from some guanidino compounds were compared. A method for estimation of arginine in serum is briefly communicated.

Expenses of this work were defrayed by Grants for the Advancement of Science delivered by the Department of Education to Prof. S. Akamatsu.

REFERENCES

- (1) Sakaguchi, S., *J. Biochem.*, **5**, 25 and 133 (1925)
- (2) Weber, C. J., *J. Biol. Chem.*, **86**, 217 (1930)
- (3) Sakaguchi, S., *J. Biochem.*, **37**, 231 (1950) and **38**, 91 (1951)
- (4) Macpherson, H. T., *Biochem. J.*, **36**, 59 (1942)
- (5) Jorpes, E. and Thorén, S., *Biochem. J.*, **26**, 1504 (1932)
- (6) Fisher, R. B., and Wilhelmi, A. E., *Biochem. J.*, **32**, 606 (1938)
- (7) Thomas, L. E., Ingalls, J. K., and Luck, J. M., *J. Biol. Chem.*, **129**, 263 (1939)
- (8) van Pilsum, F., Martin, R. P., Kito, E. and Hess, J., *J. Biol. Chem.*, **222**, 225 (1956)
- (9) Ceriotti, G. and Spandrio, L., *Biochem. J.*, **66**, 603 (1957)
- (10) Ishikawa, T., *J. Chiba Med. Soc.*, **34**, 1134 (1958), in Japanese.

Carboxylic Acids Metabolism and its Relation to Porphyrin Biosynthesis in *Rhodospseudomonas spheroides* under Light-Anaerobic Conditions*

By GORO KIKUCHI, SHOICHI ABE† and AKIRA MUTO

(From the Department of Biochemistry, Tohoku University School of Medicine, Sendai)

(Received for publication, February 28, 1961)

Rhodospseudomonas spheroides produces large amounts of porphyrin when it was grown under light-anaerobic conditions. In contrast, the dark-aerobically grown cells have only trace amounts of bacteriochlorophyll (1). Also levels of δ -aminolevulinic acid-synthesizing enzyme are far lower in dark-aerobically grown cells as compared to those in light-anaerobically grown cells (2). When, however, the dark-aerobically grown cells were transferred to the light-anaerobic conditions, the activity of δ -aminolevulinic acid-synthesizing enzyme as well as the bacteriochlorophyll content increased rapidly to levels of those in light-anaerobically grown cells. Of steps for total biosynthesis of porphyrin in *R. spheroides*, the step of δ -aminolevulinic acid synthesis seems to be most susceptible of environmental changes (3). This may be due to the fact that the δ -aminolevulinic acid is synthesized from such common sources as succinyl-CoA and glycine. We may expect that the formation of δ -aminolevulinic acid could be influenced if any significant changes were resulted in the metabolism of carboxylic acids and of amino acids by environmental changes. Considering this possibility, we have studied the metabolism of carboxylic acids, in particular of succinic acid, in *R. spheroides* under

light-anaerobic conditions.

With respect to the carboxylic acids metabolism in non-sulfur purple bacteria, there have been many papers, mostly dealing with *Rhodospirillum rubrum*, reported by Van Niel (4), Stanier (5), Ormerod (6), Elsdon (7), Kamen (8), Eisenberg (9), and else (10, 11). From the results in these papers, it appears that carboxylic acids are metabolized *via* ordinary citric acid cycle under dark-aerobic conditions, whereas under light-anaerobic conditions, the activity of citric acid cycle is suppressed and the cycle operates only partly. In this paper, we will report that the metabolism of carboxylic acids in *R. spheroides* under light-anaerobic conditions appears further to involve coupled oxidation-reduction reactions between acids of different oxidation levels which are mediated by photochemical oxidation-reduction systems. Also a discussion will be made on the significance of this characteristic pattern of carboxylic acids metabolism in the control of porphyrin biosynthesis in *R. spheroides*.

MATERIALS AND METHODS

R. spheroides, originally obtained from Dr. Van Niel, was grown light-anaerobically in medium S of Lascelles (12), usually for 72 hours at 30°C. Harvested cells were washed twice with 0.02 M phosphate buffer (pH 6.8), then suspended in 20 times the volume of 0.04 M phosphate buffer (pH 6.8) and used. Cell amounts used were measured in terms of dry weight.

Cell-free extracts of *R. spheroides* were prepared as follows: washed cells were sonicated for 4 minutes with 10 kc. Kubota Sonic oscillator; the sonicates were centrifuged at 10,000 $\times g$ for 30 minutes and resulting supernatants were used. Protein contents of cell-free extracts were assayed by the biuret method modified

* This work was aided by Grants from the Rockefeller Foundation and from the Waksman Foundation of Japan. Presented in part at the 33rd Meeting of the Japanese Biochemical Society, Tokyo, in November, 1960.

† A part of this work is taken from a thesis submitted to the Tohoku University in partial fulfillment of the requirements for the degree of Doctor of Medical Science.

by Vernon and Kamen (13).

Reactions were run at 30°C in Warburg's manometric flasks immersed in a water bath with two glass windows. Illumination was provided by five 200 watt tungsten lamps set close to and in parallel to the glass window on the back side of the water bath. Light intensities provided to the flasks were about 28,000 luxes. Anaerobiosis was obtained by using nitrogen gas as the gas phase. The gas was purified by passing it through $\text{Na}_2\text{S}_2\text{O}_4$ -KOH-solution and pyrogallol- β -anthraquinone sulfonate-KOH mixture (14).

C^{14} -labeled compounds were purchased from Daiichi Chemical Co., Tokyo. The specific radioactivity and the concentration of solutions of C^{14} -labeled compounds were made up to 0.01 mc/mmmole and 0.1 M, respectively. Other reagents used were obtained commercially.

Radioactivities of reaction products were measured by a windowless gas flow counter from Nihon Musen Irigakukenkyusho, Tokyo. When C^{14}O_2 was the marked product, 100 μ moles of Na_2CO_3 were added as the carrier and counted as BaCO_3 .

RESULTS

CO₂ Evolution from Carboxylic Acids—R. spheroides cells were illuminated anaerobically in the presence of carboxylic acids. It can be seen in Table I that fair amounts of CO_2 were evolved when fumarate, malate, and pyruvate were the substrates. When the succinate and lactate were the substrates, however, amounts of CO_2 evolved were minor. Also it was observed that rates of CO_2 evolution in systems containing any of fumarate, malate, and pyruvate as substrates were greatly diminished by the addition of the equal amount of succinate. Similar effect was observed also with lactate. It was noticed that more amounts of CO_2 were evolved when succinate was added to the system with acetate (Table I, Exp. 2). As will be seen later in DISCUSSION, it appears that the acid which liberated more CO_2 might be not the acetate but the succinate.

C¹⁴O₂ Formation from Succinate-1,4-C¹⁴—In contrast to the data presented in Table I, C^{14}O_2 formation from succinate-1,4- C^{14} under light-anaerobic conditions was increased by the addition of fumarate (Table II, Expts. 1 and 2). The results are significant particularly when we take it into considerations that, in

TABLE I

CO₂ Evolution from Carboxylic Acids Under Light-anaerobic Conditions

Reaction mixtures contained per flask 40 μ moles each of substrates and additions, 2 ml. of 0.06 M phosphate buffer (pH 6.9), and *R. spheroides* cells. Final volume was 3.0 ml.

Substrates	CO ₂ , μ l./60 min.		Dry weights of the cells used, mg.
		Added with Succinate	
Exp. 1			4.0
Succinate	6	—	
Fumarate	75	17	
Malate	68	9	
Exp. 2			7.5
Succinate	10	—	
Pyruvate	81	40	
Fumarate	70	30	
α -Ketoglutarate	11	13	
Lactate	11	—	
Acetate	22	45	
Exp. 3			6.1
Fumarate	139	—	
Lactate	65		
Fumarate plus lactate	68	—	

this case, the fumarate- C^{14} from succinate- C^{14} should have been heavily diluted with the non-labeled fumarate added. Among acids tested, oxaloacetate and acetate were the most effective ones in promoting C^{14}O_2 formation from succinate- C^{14} . The addition of lactate to the system was found to decrease the C^{14}O_2 formation. Similar results as in Table II were obtained also with citrate-1, 5- C^{14} (Table III). Cell-free extracts were used for the experiments with citrate-1,5- C^{14} . Under dark-aerobic conditions, no such effect was seen as observed under light-anaerobic conditions, regardless of substrates and additions used. The data presented in Tables I, II and III, indicate that the photochemical oxidation of acids of higher oxidation levels is suppressed by the addition of acids of lower oxidation levels, and in turn, the photochemical oxidation of acids of lower oxidation levels is promoted by the addition of acids of higher

TABLE II

$C^{14}O_2$ Formation from Succinate-1, 4- C^{14} and Effects on it of Additions of Other Non-labeled Carboxylic Acids under Light-anaerobic Conditions

The substrate and additions used were 20 μ moles each. Other conditions were the same as in Table I.

Substrate and additions	Radioactivity of CO_2		Dry weights of <i>R. spheroides</i> cells used, mg.
	c. p. m.	Relative activity, %	
Exp. 1			10.9
Succinate- C^{14}	661	100	
Plus succinate	387	59	
Plus fumarate	749	113	
Plus malate	683	102	
Plus oxaloacetate	12,009	1,820	
Exp. 2			7.2
Succinate- C^{14}	450	100	
Plus fumarate	580	130	
Exp. 3			8.2
Succinate- C^{14}	1,000	100	
Plus pyruvate	4,800	480	
Exp. 4			7.3
Succinate- C^{14}	608	100	
Plus acetate	10,341	1,700	
Exp. 5			4.8
Succinate- C^{14}	1,506	100	
Plus lactate	1,011	67	

TABLE III

$C^{14}O_2$ Formation from Citrate-1, 5- C^{14} under Light-anaerobic Conditions

R. spheroides extracts used per flask contained 3.0 mg. protein and 0.06 mg. chlorophyll.

The substrate and additions used were 20 μ moles each. Other conditions were the same as in Table I.

Substrate and additions	Radioactivity of CO_2	
	c. p. m.	Relative activity, %
Citrate- C^{14}	113	100
Plus succinate	124	110
Plus fumarate	157	139
Plus pyruvate	1,001	886
Plus lactate	150	133
Plus acetate	145	129
Plus oxaloacetate	1,766	1,560

oxidation levels. In other words, there appears to occur photochemical coupled oxidation-reduction reactions between acids of different oxidation levels.

Effects of Fluoroacetate and Sodium Bisulfite—As expected, the $C^{14}O_2$ formation from succinate-1, 4- C^{14} as well as from acetate-1- C^{14} under dark-aerobic conditions was strongly inhibited by relatively low concentrations of fluoroacetate, indicating that the succinate and acetate are metabolized through ordinary citric acid cycle. Under light-anaerobic conditions, however, the $C^{14}O_2$ formation from succinate-1, 4- C^{14} was not inhibited by the addition of as high as $1 \times 10^{-3}M$ fluoroacetate (Table IV). Also it was observed that the addition of fluoroacetate did not significantly affect the effects of acetate and oxaloacetate in promoting the CO_2 formation from succi-

TABLE IV

Effect of Monofluoroacetate upon $C^{14}O_2$ Formation from Succinate-1, 4- C^{14} and from Acetate-1- C^{14}

Substrates and other non-labeled carboxylic acids used were 20 μ moles each. Dry weights of *R. spheroides* cells used were 5.2 mg. in A, 6.8 mg. in B, Exp. 1, 9.4 mg. in B, Exp. 2, and 6.3 mg. in Exp. 3. Other conditions were the same as in Table I.

A. Under dark-aerobic conditions

Substrates and additions	Radioactivity of CO_2 c. p. m.
Acetate- C^{14}	6,682
Plus fluoroacetate, $7 \times 10^{-6} M$	630
Succinate- C^{14}	18,581
Plus fluoroacetate, $7 \times 10^{-6} M$	7,120

B. Under light-anaerobic conditions

Substrates and additions	In the absence of fluoroacetate	Added with fluoroacetate	
	Radioactivity of CO_2 , c. p. m.	Concentration of fluoroacetate, M	Radioactivity of CO_2 c. p. m.
Exp. 1			
Acetate- C^{14}	700	1×10^{-5}	634
"	"	1×10^{-3}	383
"	"	2×10^{-3}	323
Exp. 2.			
Succinate- C^{14}	770	5×10^{-5}	795
Plus pyruvate	4,800	5×10^{-5}	4,813
Plus acetate	4,707	5×10^{-5}	4,647
"	"	1×10^{-4}	4,807
Exp. 3			
Succinate- C^{14}	297	1×10^{-3}	320
Plus oxaloacetate	2,886	2×10^{-3}	2,316

nate- C^{14} , suggesting that the effects may not be due to their potencies as the acetyl-CoA donor in citric acid cycle.

The experimental results obtained with sodium bisulfite are shown in Table V. The dark-aerobic metabolism of succinate was strongly inhibited by sodium bisulfite, whereas the light-anaerobic metabolism of succinate was not inhibited but slightly increased by addition of the same concentration of bisulfite as used in dark-aerobic experiments. In the presence of bisulfite, the effect of oxaloacetate in promoting the CO_2 formation from succinate-1, 4- C^{14} was almost completely lost and also the similar effect of acetate was diminished

significantly. From the results in Table IV and V, we may conclude that the succinate is metabolized to CO_2 and probably to pyruvate mainly at stages of malate and oxaloacetate under light-anaerobic conditions.

With acetate-1- C^{14} as the substrate, however, inhibitory effects of fluoroacetate and bisulfite were more pronounced. $C^{14}O_2$ formation from acetate-1- C^{14} under light-anaerobic conditions was inhibited increasingly with increased addition of fluoroacetate. Also the light-anaerobic $C^{14}O_2$ formation from acetate-1- C^{14} was inhibited by bisulfite to almost the same extent as observed under dark-aerobic conditions. It should be noted, however, that

TABLE V

Effect of Sodium Bisulfite upon $C^{14}O_2$ Formation from Succinate-1, 4- C^{14} and from Acetate-1- C^{14}

Substrates and other non-labeled carboxylic acids used were 20 μ moles each. Dry weights of *R. spheroides* cells used were 4.8 mg. in A, and 6.1 mg. in B.

A. Under dark-aerobic conditions

Substrates and additions	Radioactivity of CO_2 c. p. m.
Acetate- C^{14}	5,301
Plus bisulfite, $5 \times 10^{-3}M$	97
Succinate- C^{14}	10,315
Plus bisulfite, $5 \times 10^{-3}M$	2,367

B. Under light-anaerobic conditions

Substrate and additions	Radioactivity of CO_2 , c.p.m.	
	Without bisulfite	Added with $1 \times 10^{-3}M$ bisulfite
Succinate- C^{14}	330	501
Plus acetate	6,800	2,880
Plus fumarate	683	563
Plus oxaloacetate	1,726	674

the extent of inhibition by fluoroacetate did not exceed over approximately 60% even when the system was added with as high as $2 \times 10^{-3}M$ fluoroacetate. Further studies are needed as to how the acetate is metabolized under light-anaerobic conditions.

Effects of Other Substances—The light-anaerobic metabolism of succinate was not significantly inhibited by $1 \times 10^{-3}M$ KCN (Table VI, A). The data are in accord with our unpublished observation that the oxidation of succinate by chromatophores as measured by oxygen uptake under light-aerobic conditions was not inhibited by KCN.

The $C^{14}O_2$ formation from succinate-1, 4- C^{14} was almost completely inhibited by the addition of $1 \times 10^{-4}M$ methylene blue (Table VI, B). It appears that the addition of methylene blue provided a "shunt" between photo-oxidants and photoreductants as suggested by Vernon (15, 16), thus obviating the photochemical oxidation of succinate.

Light-anaerobic metabolism of succinate

TABLE VI

Effects of Various Substances upon $C^{14}O_2$ Formation from Succinate-1, 4- C^{14} under Light-anaerobic Conditions

Carboxylic acids used were 20 μ moles each. Dry weights of *R. spheroides* cells used were 7.6 mg. in A, 7.7 mg. in B, and 8.2 mg. in C. Other conditions were the same as in Table I.

A. Effect of KCN

Substrate and addition	Radioactivity of CO_2 , c.p.m.	
	Without KCN	Added with $1 \times 10^{-3}M$ KCN
Succinate- C^{14}	443	345
Plus acetate	3,267	3,251

B. Effect of methylene blue

Substrate and additions	Radioactivity of CO_2 , c. p. m.
Succinate- C^{14}	444
Plus methylene blue, 0.3 μ mole	16

C. Effects of thioctic amide and oxidized glutathione

Substrate and additions	Radioactivity of CO_2 c. p. m.
Succinate- C^{14}	508
Plus thioctic amide, 1.5 μ moles	917
Plus oxidized glutathione, 1.5 μ moles	659

was increased by the addition of a minute amount of thioctic amide. A similar effect was seen also with oxidized glutathione, though to less extents. It is not clear at present whether the observed effect of thioctic amide was due to its positive action in photochemical reactions as suggested by Calvin *et al.* (17, 18), or it was just because the thioctic amide acted as a hydrogen acceptor.

Chromatographic Analysis of Succinate Metabolism—Reactions with cell-free extracts were run under conditions as described in Fig. 1. When succinate- C^{14} was the marked substrate, non-labeled citrate was added as the carrier. When citrate- C^{14} was the marked substrate, non-labeled succinate was added as the carrier. Reactions were stopped after 60 minutes run by sulfuric acid and organic acids in the reaction mixtures were extracted with ether for 72 hours by using Kutscher-Stendel's

liquid-liquid extractor. The acids thus obtained were subjected to ascending zone chromatography on Toyo Filter paper, No. 50 (50×40 cm.) using *n*-butanol-propionic acid-water mixture (20) as the solvent. After completion of chromatography, the papers were cut into 2 cm. wide strips in parallel to the starting line and each strip was eluted 5 times with hot water. Each eluate was condensed by evaporation under reduced pressure, transferred quantitatively to a planchet, and dried under an infra-red lamp. Radioactivities of those samples are compared in Fig. 1. As seen from the figure, under dark-aerobic con-

to succinate under the same conditions. The data indicate that under light-anaerobic conditions, the citric acid cycle does not operate efficiently, so that succinate is hardly metabolized *via* the citric acid cycle.

DISCUSSION

From the data presented, it was suggested that under light-anaerobic conditions, the citric acid cycle does not operate regularly and instead there occur coupled oxidation-reduction reactions between carboxylic acids of oxidatively different levels. The metabolism of succinate appears to proceed mainly through such coupled reactions. As a simple case of light-anaerobic metabolism of carboxylic acids, a dismutation reaction of fumarate-succinate is proposed schematically in Fig. 2. When the fumarate or malate is photochemically metabolized into CO_2 and pyruvate, hydrogens from the substrates would be transferred to photooxidant $[\text{O}]$ to give H_2O . Then it is expected that the equivalent amounts of photoreductant $[\text{H}]$ could reduce CO_2 , pyruvate, and fumarate itself, giving rise to the formation of lactate, succinate and the CO_2 fixation. Now, when succinate was added to the system in which fumarate was the substrate, the photooxidant would be consumed predominantly by hydrogens from succinate, so that the oxidation of fumarate or malate would be suppressed as evidenced by the reduced formation of CO_2 from fumarate or malate as shown in Table 1. Also in such a case more amounts of fumarate would be reduced to succinate. When succinate was the sole substrate, in turn, we may expect that some of the fumarate which were resulted by the succinate oxidation would concomitantly be reduced to succinate by photoreductants, so that the probability of breakdown of the given succinate molecules beyond fumarate step would become less. This may explain why the CO_2 formation from succinate was so small (Table I). If these were the cases, it can be expected that the C^{14}O_2 formation from succinate- C^{14} would be increased when the system was added with fumarate or other carboxylic acids of higher oxidation levels

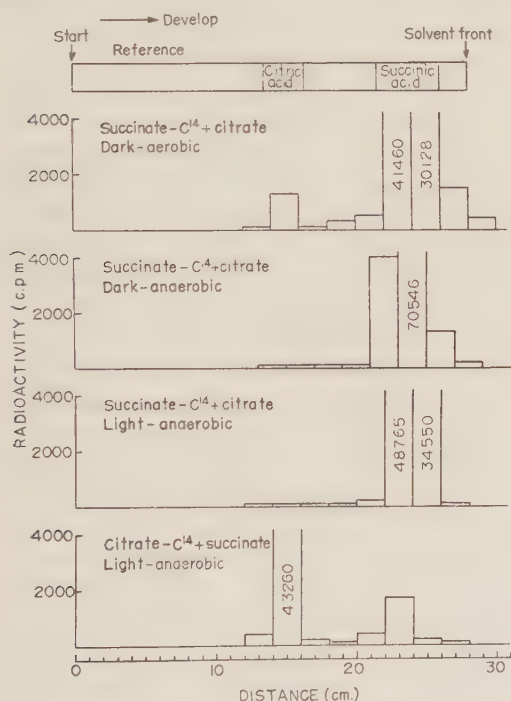


FIG. 1. Chromatography of reaction products from succinate-1, 4- C^{14} and from citrate-1, 5- C^{14} .

10 μmoles of C^{14} -labeled substrates and 50 μmoles of non-labeled carrier acids were incubated with *R. spheroides* extracts for 60 minutes at 30°C under conditions as denoted in the figure.

ditions, substantial amounts of citrate were formed from succinate. In contrast, under light-anaerobic conditions, practically no citrate was formed from succinate, although fair amounts of citrate could be metabolized

such as oxaloacetate, acetate and pyruvate. This happened actually as shown in Table II. As pointed out previously, it was noted in Table I that more amounts of CO_2 were evolved when succinate was added to the system with acetate. It appears now that the increment of CO_2 evolved over the control value might come not from acetate but from succinate. We have observed also that the C^{14}O_2 formation from acetate-1- C^{14} as well as from acetate-2- C^{14} under light-anaerobic conditions was not increased but rather slightly decreased by the addition of succinate.

The mentioned coupled oxidation-reduction appeared to function also in the metabolism of citrate (Table III). Therefore we may assume that it represents a general pattern of carboxylic acids metabolism in *R. spheroides* under light-anaerobic conditions.

Data have been accumulating also from other laboratories suggesting the characteristic nature of carboxylic acids metabolism in photosynthetic bacteria. Frenkel (19) observed with chromatophores of *R. rubrum* that the photoreduction of DPN under anaerobic conditions proceeded only when a small amount of succinate was added to the reaction system, and that the reaction was inhibited about 80 per cent by $1 \times 10^{-3} M$ malonate. Vernon (16), on the other hand, reported that the reaction of photooxidation of reduced cytochrome C with *R. rubrum* chromatophores under anaerobic conditions required the presence of fumarate in the system. These data suggest that oxidation-reductions of succinate and fumarate are intimately coupled to photo-oxidation-reduction reactions.

It is important to note that CO_2 can also act as a hydrogen acceptor. Elsdon and Ormerod (7) reported that the light-anaerobic metabolism of succinate in *R. rubrum* increased as much as three times when the gas phase contained CO_2 . Also it has been reported by Kamen *et al.* (8) and by Ormerod (6) that C^{14}O_2 incorporation into *R. rubrum* cells under light-anaerobic conditions was suppressed by the addition of acetate. In the latter case, CO_2 and acetate may be competing with each other for photoreductants. Accord-

ing to Ormerod, also the incorporation of C^{14}O_2 was increased about three times when succinate was added to the system. It is worthwhile to note that the experiments by Ormerod were carried out using $33.9 \mu\text{moles C}^{14}\text{O}_2$ in the gas phase—in other words, in the presence of sufficient amounts of the hydrogen acceptor. In contrast, our experiments were run under nitrogen without CO_2 . It is not clear how much CO_2 could be fixed under our experimental conditions. Yet, it can not be overlooked that the C^{14}O_2 formation from succinate- C^{14} was increased as much as 18 times by oxaloacetate, and 17 times by acetate, while the succinate metabolism was increased only three times by the presence of CO_2 as cited above. Also ratios of C^{14}O_2 fixation in the presence of acetate to endogeneous values as calculated from Ormerod's data, were found to fall in the range of 0.8 to 0.3. Judging from these figures, we are inclined to assume that the observed 17 to 18 times increases in C^{14}O_2 formation from succinate- C^{14} could not be fully accounted for by the decreased C^{14}O_2 fixation which would concomitantly occur in the presence of acetate or oxaloacetate.

That the metabolism of carboxylic acids involves coupled oxidation-reduction reactions appears to provide a favorable condition for the porphyrin biosynthesis in *R. spheroides* under light-anaerobic conditions. It has been found by Lascelles (12) that the porphyrin formation by *R. spheroides* was highest when the cells were incubated in Lascelles' medium I which contained both fumarate and α -ketoglutarate as carbon sources. The medium contained also glycine. We have tried similar experiments and confirmed her findings*. The advantage of medium I in the biosynthesis of porphyrin could be explained in terms of the increased supply of succinyl-CoA. Namely, accompanying to the oxidation of α -ketoglutarate to succinyl-CoA, fumarate could simultaneously be reduced to succinate. The oxidation of fumarate, in turn, would be accompanied by the concomitant reduction of fumarate to succinate as a result of pre-

* Collaborated by Dr. Hyogo Sinohara.

sumed dismutation as schematized in Fig. 2. By this way, the carboxylic acids metabolism on either side of succinyl-CoA in the citric acid cycle would give rise to the increased supply of succinyl-CoA. On similar basis, we may expect that, whatever the carbon sources

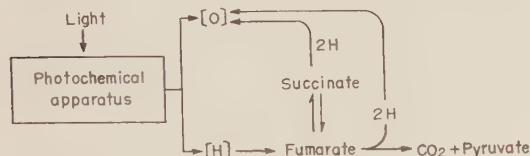


FIG. 2. Scheme for the presumed interconversion of succinate and fumarate under light-anaerobic conditions.

may be, more succinyl-CoA would be produced under light-anaerobic conditions than under dark-aerobic conditions, which consequently would give rise to the increased synthesis of δ -aminolevulinic acid. It remains to be elucidated, however, whether such metabolic change as mentioned above could induce the adaptive formation of δ -aminolevulinic acid-synthesizing enzyme, although it has been found that levels of the enzyme are much higher in light-anaerobically grown cells.

SUMMARY

Studies were made on the metabolism of carboxylic acids, in particular of succinic acid, in *Rhodospseudomonas spheroides* under light-anaerobic conditions.

1. Fair amounts of CO_2 were evolved when the cells were illuminated anaerobically in the presence of carboxylic acids of higher oxidation levels such as fumarate, malate and pyruvate, while the amounts of CO_2 evolved from acids of lower oxidation levels such as succinate and lactate were minor. Also the CO_2 evolution from acids of higher oxidation levels was significantly suppressed by the addition of acids of lower oxidation levels.

2. C^{14}O_2 formation from succinate-1,4- C^{14} under light-anaerobic conditions was greatly increased by the addition of other acids of higher oxidation levels, while it was decreased by the addition of lactate. Similar results

were obtained also with respect to C^{14}O_2 formation from citrate-1,5- C^{14} .

3. C^{14}O_2 formation from succinate-1,4- C^{14} under light-anaerobic conditions was not inhibited by sodium fluoroacetate and sodium bisulfite.

From these results, it was proposed that, under light-anaerobic conditions, there seems to occur photochemical coupled oxidation-reduction reactions between acids of higher oxidation levels and of lower oxidation levels. Also it was suggested that, as a result of the coupled reactions, the supply of succinyl-CoA to the porphyrin-synthesis system could be increased, which consequently would give rise to the increased porphyrin formation in *R. spheroides* under light-anaerobic conditions.

REFERENCES

- (1) Cohen-Bazire, G., Sistrom, W. R., and Stanier, R. Y., *J. Cell. Comp. Physiol.*, **49**, 25 (1957)
- (2) Kikuchi, G., Kumar, A., Talmage, P., and Shemin, D., *J. Biol. Chem.*, **233**, 1214 (1958)
- (3) Lascelles, J., *Biochem. J.*, **72**, 508 (1959)
- (4) Van Niel, C. B., *Advances in Enzymol.*, **1**, 263 (1941)
- (5) Stanier, R. Y., Doudoroff, M., Kunisawa, R., and Contopoulou, R., *Proc. Natl. Acad. Sci., U. S.*, **45**, 1246 (1959)
- (6) Ormerod, J. G., *Biochem. J.*, **64**, 373 (1956)
- (7) Elsdon, S. R., and Ormerod, J. G., *Biochem. J.*, **63**, 691 (1956)
- (8) Glover, J., Kamen, M. D., and Van Genderen, H., *Arch. Biochem. Biophys.*, **35**, 384 (1953)
- (9) Eisenberg, M. A., *J. Biol. Chem.*, **203**, 815 (1953)
- (10) Clayton, R. K., *Arkiv für Mikrobiol.*, **22**, 180, 195, 204 (1955)
- (11) Crook, P. G., and Lindstrom, E. S., *Canad. J. Microbiol.*, **2**, 427 (1956)
- (12) Lascelles, J., *Biochem. J.*, **62**, 78 (1956)
- (13) Vernon, L. P., and Kamen, M. D., *Arch. Biochem. Biophys.*, **44**, 298 (1953)
- (14) Lundquist, F., Fugmann, U., Kläming, E., and Rasmussen, H., *Biochem. J.*, **72**, 409 (1959)
- (15) Vernon, L. P., and Ash, O. K., *J. Biol. Chem.*, **234**, 1878 (1959)
- (16) Vernon, L. P., *J. Biol. Chem.*, **234**, 1883 (1959)
- (17) Bartrop, J. A., Calvin, M., and Hayes, P. M., *J. Am. Chem. Soc.*, **76**, 1760 (1954)
- (18) Bradley, D. F., and Calvin, M., *Arch. Biochem. Biophys.*, **53**, 99 (1954)
- (19) Frenkel, A. W., 'Brookhaven Symposia in Biology,' No. 11, The photochemical apparatus (1958), p. 276.
- (20) Calvin, M., and Benson, A. A., *Science*, **109**, 140 (1949)

Enzymatic Synthesis of the Methyl Group of Methionine

III. Spectral and Electrophoretic Studies of the Prosthetic Group of the B₁₂ Enzyme*

By SHIGEYUKI TAKEYAMA** and JOHN M. BUCHANAN

(From the Division of Biochemistry, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, U.S.A.)

(Received for publication, April 12, 1961)

During the last few years several enzymatic reactions have been shown to involve the participation of cobamide derivatives (1-6). These findings were aided by the discovery by Barker *et al.* of a coenzyme form of vitamin B₁₂ or its derivatives in microbial cells (1, 7-9). This coenzyme derivative, in which the cyano group of cyanocobamides is replaced by an adenine-containing moiety, is the only active form in several of the cobamide-dependent reactions reported (1, 4-6).

However, in the enzymatic system in *Escherichia coli* concerned with the biosynthesis of methionine-methyl, vitamin B₁₂ is utilized (3, 10). Although the DBC*** coenzyme has been shown to be as active as vitamin B₁₂ in stimulating methionine synthesis in 'crude' extracts of *E. coli*, there is substantial evidence that the cobamide coenzyme itself is not the active derivative in this system (3, 11). In fact, when a system consisting of partially purified enzymes is used, the DBC coenzyme has been shown to be not only much less active than vitamin B₁₂ but also serves as an inhibitor of the action of this latter com-

pound. It is now known that cyanocobalamin must be converted into an active form in the course of its combination with the apoenzyme and that the DBC coenzyme is poorly utilized when the apoenzyme has been purified by previously reported procedures (3). These observations led the authors to the conclusion that the DBC coenzyme may not be the true coenzyme form of vitamin B₁₂ in the methionine-synthesizing system.

This conclusion may also apply to other enzymatic systems which do utilize the cobamide coenzymes. In previous investigations the cobamide derivatives discovered by Barker *et al.* have been assigned a coenzymatic role by virtue of their ability to stimulate enzymatic reactions by combination with apoenzyme from which cobamide compounds have been removed. These investigations do not necessarily describe the structure of the prosthetic group of a cobamide enzyme since the cobamide coenzyme may have undergone alterations in the process of becoming bound to the apoenzyme. Since the cobamide coenzymes of Barker *et al.* were isolated from intact cells, these derivatives may represent a metabolic form of free cobamides in the cells rather than the actual prosthetic group of the cobamide-containing enzymes.

In this communication the cobamide-containing enzyme involved in methionine-methyl biosynthesis has been purified from the cells of *E. coli* mutant 113-3 grown on vitamin B₁₂ and limiting methionine and a study has been made of a cobamide derivative isolated directly from the enzyme. This

* This work was supported by grants-in-aid from the National Science Foundation.

** Karl Compton Fellow in Biochemistry of the Nutrition Foundation (1958-1961) on leave of absence from Tanabe Seiyaku Co., Ltd., Osaka, Japan.

*** The abbreviations used are: DBC, dimethylbenzimidazolylcobamide; AC, adenylcobamide; folate-H₄, tetrahydrofolic acid; DEAE, diethylaminoethyl; KPO₄ (1:1), (2:1) or (4:1), buffer in which the ratio of K₂HPO₄ to KH₂PO₄ is 1:1, 2:1 or 4:1, respectively.

cobamide compound (tentatively called "B₁₂ coenzyme M") appears to be neither vitamin B₁₂ nor the DBC coenzyme.

EXPERIMENTAL AND RESULTS

Several of the materials and methods used in this investigation have been described in the previous papers of this series (3, 12). Included are descriptions of the mutant strain 113-3 of *E. coli* and its culture, the procedures used for determining the formation of B₁₂ enzyme from cobamide compounds and apoenzyme, and the assay method for methionine synthesis *in vitro*.

Protamine sulfate (salmine) was purchased from Mann Research Laboratories, Inc., N. Y. Alumina was washed with distilled water several times and was dried before use. Absorption spectra were taken with Cary Recording Spectrophotometer Model 11 M or Beckman Spectrophotometer Model DU in the case of anaerobic measurements.

Purification of B₁₂ Enzyme—Several modifications in the preparation of B₁₂ enzyme have been made over the method previously described (12).

1. Cell Culture of E. coli 113-3: Cells of *E. coli* 113-3 were grown in 15 liter carboys as described previously (12) except that vitamin B₁₂ was added at the concentration of 10⁻⁸ M instead of 10⁻⁹ M. This increase in vitamin B₁₂ concentration brought about an almost two-fold increase in the specific activity of the B₁₂ enzyme of cell-free extracts. Raising the level of vitamin B₁₂ to 10⁻⁷ M did not result in further marked change in the amount of B₁₂ enzyme produced. Cells harvested from fifteen such carboy cultures were pooled (total wet weight, 1216 g.) and stored frozen. In a previous paper (3) we have shown that, from a culture of *E. coli* 113-3 grown on 10⁻⁹ M Co⁶⁰ labeled vitamin B₁₂, thirty-six per cent of the soluble radioactivity may be recovered as B₁₂ enzyme after subsequent ammonium sulfate fractionation and chromatography on a hydroxylapatite column and that no other major radioactive fractions are observed. Therefore the radioactivity

may be used as a means of assay of the B₁₂ enzyme through subsequent purification steps.

2. Preparation of Cell-free Extracts: Extracts have been prepared by grinding the cells with alumina rather than by rupturing them in the Hughes press. The specific activity of B₁₂ enzyme and the yield of soluble protein of the extracts are the same by either procedure. One part of the cells was mixed with three parts (in weight) of alumina and ground in a mortar at 3°C. Grinding was continued until the mixture became homogenous and pasty. The ground mixture was extracted with five cell volumes of 0.03M KPO₄ (2:1)*** and the extract was obtained by centrifugation at 15,000×g for 40 minutes. Such extracts were pooled.

3. Protamine Treatment: Another change in the standard fractionation procedure involves the use of protamine to remove the nucleic acids which interfere with the subsequent fractionation. It was found that B₁₂ enzyme was also precipitated with nucleoproteins, particularly at lower pH values and at relatively low concentration of phosphate buffer. However, B₁₂ enzyme does not precipitate until a certain amount of protamine is added, although the precipitation of nucleoproteins takes place instantly. Therefore, a set of conditions could be devised in which stepwise addition of protamine brought down most of nucleoproteins leaving B₁₂ enzyme in solution. Then, with further addition of protamine, precipitation of B₁₂ enzyme could be effected. B₁₂ enzyme in the second precipitate could be brought into solution by suspending the precipitate in a phosphate buffer of higher concentration and higher pH. In this way one can at the same time remove nucleoproteins and achieve a three- to four-fold purification of the enzyme with a yield of about 70 per cent.

The specific conditions for this procedure are as follows: the extracts of alumina-ground cells were diluted with 0.03 M KPO₄ (2:1) to yield a protein concentration of 10 mg. per ml. and were then mixed with an extract obtained in the same way from the cells

grown on 10^{-9} M radioactive vitamin B₁₂ (110mg. protein, 272,000 c.p.m.). A 2 per cent solution of salmine was added slowly with vigorous stirring in a ratio of 0.1 ml. per ml. of the diluted extract at 0–2°C. The outlet of the vessel containing the salmine solution was kept immersed in the enzyme solution during the time of addition (30 to 40 minutes). The stirring was continued for 15 minutes after the addition. The resulting stringy precipitate was centrifuged down with the Stock centrifuge at $5000\times g$ for 30 minutes. The precipitate was discarded and the supernatant solution was further fractionated. To this supernatant solution was added 2 per cent salmine solution in exactly the same amount and in the same manner as in the first protamine treatment. The resulting precipitate was collected by centrifugation and was extracted with 520 ml. of 0.2 M KPO₂ (4:1) and centrifuged at $15,000\times g$ for 15 minutes. The residue was washed with 260 ml. of the same buffer. The supernatant solution and the wash were combined and used for fractionation with ammonium sulfate.

4. *Ammonium Sulfate Precipitation*: The above fraction was diluted with 0.2 M KPO₄ (4:1) to yield a protein concentration of about 15 mg. per ml. Saturated ammonium sulfate solution (approximately 1/3 of the volume) and solid ammonium sulfate were added in a stepwise manner to bring the solution to 35 per cent of saturation. The precipitate was discarded. The supernatant solution was brought to 50 per cent of saturation with an additional amount of ammonium sulfate. The supernatant solution was discarded and the precipitate was dissolved in 150 ml. of 0.03 M KPO₄ (2:1) and dialyzed extensively against 0.01 M KPO₄ (1:1).

5. *Calcium Phosphate Gel Treatment*: The dialyzed 35–50 per cent ammonium sulfate fraction was diluted with 0.01 M KPO₄ (1:1) to bring the protein concentration to 15 mg. per ml. Calcium phosphate gel was added at the ratio (mg. gel/mg. protein) of 1.67 with stirring. The mixture was stirred for 15 minutes longer and centrifuged. Proteins adsorbed on the gel were eluted twice with

250 ml. of 0.03 M KPO₄ (4:1) and the eluates were combined.

6. *DEAE-Cellulose Column Chromatography*: The eluate from the gel was passed through a column (3.4×30.5 cm.) of DEAE-cellulose which had been equilibrated with 0.03 M KPO₄ (4:1). A linear gradient method was used for elution in two steps, with 450 ml. of 0.05 M KPO₄ (1:1) in the mixer and 450 ml. of 0.25 M KPO₄ (1:1) in the reservoir at the first step, and with 450 ml. of 0.25 M KPO₄ (1:1) in the mixer and 450 ml. of 0.5 M KPO₄ (1:1) in the reservoir at the second step. Fractions of 22 ml. were collected at the rate of one fraction per 6 minutes. Radioactive fractions (Nos. 35 to 57) were pooled and dialyzed against 8 liters of water overnight. Dialysis was continued against 8 liters of 0.05 M KPO₄ (4:1) for 3 hours.

7. *Hydroxylapatite Column Chromatography*: The dialyzed eluate from the DEAE-cellulose column was passed through a column (4.5×10.5 cm.) of hydroxylapatite which had been equilibrated with 0.005 M KPO₄ (4:1). Elution was carried out in a stepwise manner with 120 ml. each of 0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 M KPO₄ (4:1). Fractions of 20 ml. (six fractions for each concentration of phosphate buffer) were collected. As the elution proceeded, two faintly brown diffuse bands were visible on the column. Radioactivity was eluted along with the two bands appearing in two adjacent peaks. The first peak appeared at the 5th fraction after addition of 0.04 M buffer and the second peak appeared at the 4th fraction after addition of 0.05 M buffer. Fractions 4, 5 and 6 obtained after addition of 0.04 M phosphate buffer and fractions 1 and 2 obtained after adding 0.05 M phosphate buffer were pooled (Fraction I). The third and fourth fractions (Fraction II) and the fifth and sixth fractions (Fraction III) of 0.05 M buffer were pooled separately, because Fraction III was contaminated with a yellow colored material that would interfere with subsequent spectrophotometric measurements of B₁₂ enzyme in the other fractions.

Each radioactive fraction was assayed

TABLE I
Purification of B₁₂ Enzyme

Fractions	Total protein mg.	Radioactivity				Enzyme activity			
		Total c.p.m. $\times 10^{-3}$	Over-all recovery per cent	Specific Activity c.p.m./mg.	Over-all purification fold	Total m μ mole $\times 10^{-6}$	Over-all recovery per cent	Specific activity m μ mole/mg.	Over-all purification fold
Alumina extract	77,970	272.0	(100)	3.5	(1)	110.0	(100)	1,410	(1)
Protamine precipitate	15,950	192.0	71	12.0	3.4	77.6	71	4,870	3.5
Ammonium sulfate frac- tion 35-50 per cent	8,640	146.0	53	16.9	4.8	71.8	65	8,310	5.9
Calcium phosphate gel eluate	2,410	132.6	49	55.0	15.7	38.1	35	15,820	11.2
DEAE-cellulose No. 35-57	1,050	80.8	30	77.0	22.0	28.8	26	27,400	19.4
Hydroxylapatite Fraction I	186	49.0	18	264	75	17.7	16	95,300	68
Fraction II	51	14.4	5	282	81	4.3	4	77,300	55
Fraction III	53	7.7	3	145	41	1.8	2	34,600	25
Total of Fractions I, II and III	290	71.1	26	245	70	23.8	22	82,100	58

for its enzymatic activity by a previously reported method (12). Results of the purification are summarized in Table I. The reason for the small discrepancy in the purification pattern as measured by radioactivity and enzyme activity could not be explained.

Absorption Spectrum of B_{12} Enzyme—The major radioactive fraction (Fraction I) of the hydroxylapatite column chromatography was dialyzed extensively against water and lyophilized. The dialysis and lyophilization did not impair its enzymatic activity. The lyophilized preparation had a brownish-orange color when dissolved in a small amount of water. Charcoal treatment of B_{12} enzyme has been known not to diminish its enzymatic activity (3). The charcoal treatment did not cause any significant changes in the absorption spectrum of the enzyme solution. The spectrum of B_{12} enzyme after charcoal treatment is given in Fig. 1 (Curve A). There is

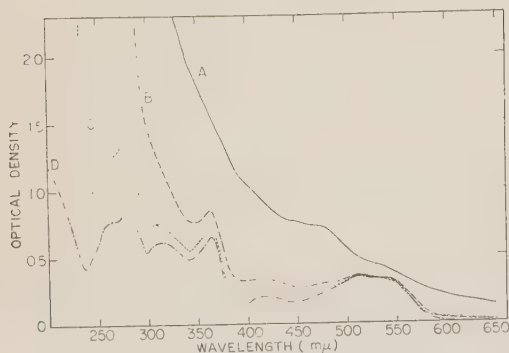


FIG. 1. Absorption spectra of B_{12} enzyme and B_{12} coenzyme M. A, B_{12} enzyme solution; B, ethanol extract of B_{12} enzyme; C and D, B_{12} coenzyme M after the first and second electrophoresis, respectively. The spectrum of each preparation was corrected to the same concentration of radioactivity (23,500 c.p.m./ml., approximately 3.9×10^{-2} mM).

a conspicuous absorption plateau around 470 $m\mu$ where vitamin B_{12} has no absorption peak, whereas there is no conspicuous absorption at 360 $m\mu$ and 550 $m\mu$ where vitamin B_{12} has characteristic absorption peaks. It is recalled that vitamin B_{12r} (13, 14), the brown-colored reduction product of vitamin B_{12} , and AC coenzyme (1) show a similar charac-

teristic in their absorption spectra. Recently, Johnson and Shaw reported that DBC coenzyme was paramagnetic and suggested that the coenzyme could be formulated as a cobaltous rather than a cobaltic complex (15). Although the spectrum of DBC coenzyme (9) is different from that of AC coenzyme or vitamin B_{12r} , the characteristic high sharp peak around 360 $m\mu$ of vitamin B_{12} and other related cobaltic cobamides is replaced by a peak of much less intensity at a somewhat longer wavelength (Fig. 5).

Ferricyanide reversibly oxidizes hemoglobin (Fe^{++}) converting it to methemoglobin (Fe^{+++}). Addition of about one molar excess of potassium ferricyanide to B_{12} enzyme solution at pH 7.0 did not cause any spectral change.

Heating of a dilute solution of B_{12} enzyme at 80°C for 5 minutes does not cause precipitation of denatured proteins. Spectra of a solution of B_{12} enzyme were compared before and after heating of the solution and are shown in Fig. 2. Although the spectral

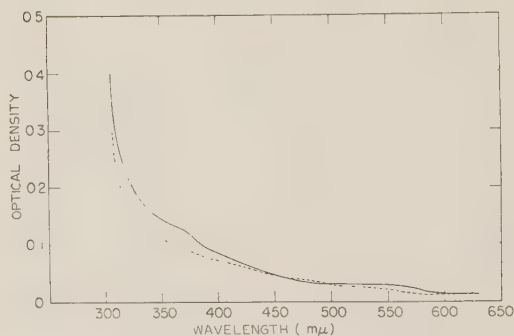


FIG. 2. Effect of heating on the spectrum of B_{12} enzyme. A solution of B_{12} enzyme (1280 c.p.m./ml., approximately 2.1×10^{-3} mM) was heated at 80°C for 5 minutes. The spectra were taken before (broken line) and after (solid line) the heating.

change after heating is slight because of the low net absorbancy of the spectra, one can see a noticeable rise at 360 and 550 $m\mu$ and disappearance of the absorption plateau at 480 $m\mu$.

Non-enzymatic Conversion of Vitamin B_{12} in the Presence of Homocysteine and FAD—In one

of the previous papers from this laboratory, the enzymatic formation of B_{12} enzyme from its apoenzyme and vitamin B_{12} was reported (3). The optimal formation of the holoenzyme required the presence of homocysteine, FAD, tetrahydrofolic acid, ATP, and Mg ion. Although homocysteine was added as a sulfhydryl agent which protected the apoenzyme from inactivation, the roles of other components were not understood.

After the spectrum of B_{12} enzyme was observed, it was anticipated that during the incubation in which the B_{12} enzyme was formed there might be a spectral change of vitamin B_{12} added in the incubation mixture. Although a spectral change took place as the incubation proceeded, it was found that only homocysteine could cause a slow spectral change in 0.05 M KPO_4 (4:1) buffer at pH 7.5 (Fig. 3). Sulfhydryl compounds are known to reduce vitamin B_{12} in alkaline solution (14, 16). When FAD was added to the mixture of vitamin B_{12} and homocysteine, FAD was reduced as evidenced by the sharp decrease of optical density at 450 $m\mu$,

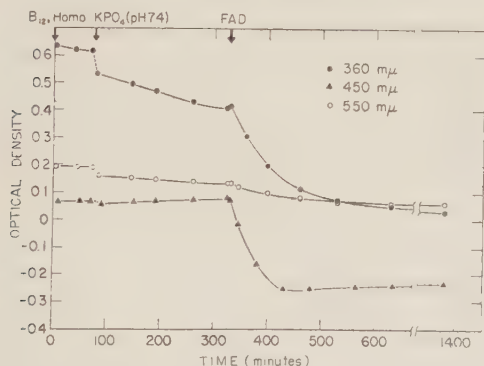


FIG. 3. Spectral change of the mixture of vitamin B_{12} , FAD and homocysteine. The reaction mixture contained 0.02 mM vitamin B_{12} and 10 mM homocysteine at the start of the reaction. 50 mM KPO_4 (4:1) and 0.04 mM FAD were added as indicated on the graph. The experiment was carried out at room temperature under nitrogen in a Thunberg-type silica cuvette. In a control cuvette additions were made in the same way except that vitamin B_{12} was omitted. Differences in optical densities between the two solutions were read and plotted.

whereas the reduction did not take place significantly in the absence of vitamin B_{12} (Fig. 3). Furthermore, the absorbancy at 360 $m\mu$ also decreased as rapidly as that at 450 $m\mu$. Since both vitamin B_{12} and FAD have high absorption at this wavelength, the interpretation of the decrease of absorbancy at 360 $m\mu$ may be less straight-forward. However, there was a more rapid decrease of the absorbancy at 550 $m\mu$ in the presence of FAD than in its absence. Since FAD has no absorption at this wavelength, this decrease in the absorbancy must be attributed to a transformation of vitamin B_{12} . Such a spectral change was not observed in a mixture of FAD and vitamin B_{12} without homocysteine. At the end of the experiment the spectrum of the reaction mixture was taken as measured against a water blank and was compared with the spectrum of a mixture of FAD and vitamin B_{12} in 0.05 M KPO_4 (4:1) buffer (Fig. 4). The spectrum has the

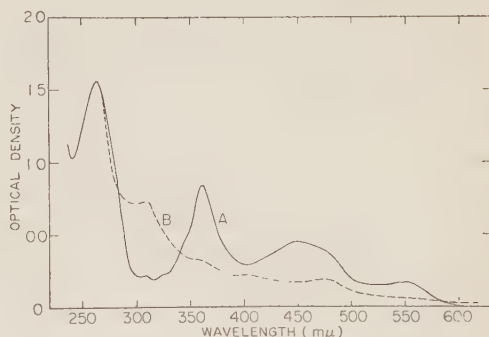


FIG. 4. Transformation of the spectrum of the mixture of vitamin B_{12} and FAD after the reaction with homocysteine. A, spectrum of a mixture of 0.02 mM vitamin B_{12} and 0.04 mM FAD in 50 mM KPO_4 (4:1); B, spectrum of the same mixture after the reaction with 10 mM homocysteine at the end of the experiment described in Fig. 3.

characteristic feature of vitamin B_{12} . If the role FAD plays in this reaction is the acceleration of reduction of vitamin B_{12} to a cobaltous cobamide and if the cobalt in B_{12} enzyme is divalent as has been suggested by its spectrum, the effect of FAD on the formation of B_{12} enzyme from its apoenzyme and

vitamin B₁₂ *in vitro* is understandable.

Isolation of a Cobamide Coenzyme from B₁₂ Enzyme—Barker *et al.* employed warm 80% ethanol to extract their coenzymes from bacterial cells (1, 9). The same procedure was applied to a purified preparation of radioactive B₁₂ enzyme. When such an extract was evaporated to dryness and subjected to electrophoresis in 0.5 *M* acetic acid, a major radioactive band (B₁₂ coenzyme M) moved towards the anode. The distance from vitamin B₁₂, which does not move electrophoretically at the pH of the solvent used, was 1.8 cm. after 2 hours electrophoresis at 2 kilovolts across 56 cm. on Whatman No. 52 filter paper. In the same solvent system Barker's coenzymes move towards the cathode relatively rapidly (1, 9).

Since B₁₂ enzyme is the major fraction of cobamide derivatives in *E. coli* 113-3 grown on 10⁻⁹ *M* vitamin B₁₂, (3), it was hoped that the prosthetic group of B₁₂ enzyme could be isolated directly from the cells. In order to increase the yield of B₁₂ enzyme, cells were grown in the presence of 10⁻⁸ *M* unlabeled vitamin B₁₂. To 150 g. of cells from a 30 liter culture grown on 10⁻⁸ *M* vitamin B₁₂, cells from a 250 ml. culture grown on 10⁻⁹ *M* radioactive vitamin B₁₂ were added and mixed. The ethanol extract of the cells was prepared and concentrated according to the procedure employed by Barker *et al.* (1). The subsequent treatment of the concentrate with Dowex resins (1) revealed that almost all the radioactivity had been converted by the cells to cationic compounds. One half of the concentrate of ethanol extract was passed through a charcoal bed (0.8×0.5 cm. column of 1:1 mixture (100 mg.) of Norit and Celite). The charcoal column was washed with 5 ml. each of water, 6% phenol and water successively. A radioactive pink eluate was obtained by putting 5 ml. of hot 65% ethanol through the charcoal column. The ethanol eluate was evaporated to dryness and subjected to electrophoresis on Whatman 3 MM paper in 0.5 *M* acetic acid (2 kilovolts across 70 cm. for 2 hours). Three colored bands were separated, one of which was unreacted

vitamin B₁₂. The other two were at 1.8 (A band) and 5.1 (B band) cm. from vitamin B₁₂ towards the cathode. On the other hand, radioactivity measured by the extent of blackening of radioautographic film was divided into four bands. The strongest radioactivity appeared on the A band. The B band had some radioactivity. Another band (C) of approximately equal radioactivity appeared between the A and B bands (4.2 cm. from vitamin B₁₂). But the band C had no color. The vitamin B₁₂ band had no radioactivity, an indication that vitamin B₁₂ was all converted to some other derivatives when cells were grown at 10⁻⁹ *M* vitamin B₁₂. The fourth and least radioactive band appeared at 4.4 cm. from vitamin B₁₂ migrating towards the anode, where B₁₂ coenzyme M would normally be found under the conditions of the electrophoresis. Since the yield of B₁₂ coenzyme M is so small, this method of isolating B₁₂ coenzyme M directly from the cells was abandoned without further attention to characterization of various bands.

It was found that electrophoresis of the ethanol extract of B₁₂ enzyme at neutral pH gave better purification of B₁₂ coenzyme M from fluorescent impurities. The following is the procedure for purification of B₁₂ coenzyme M from a B₁₂ enzyme preparation described in the previous section.

One of the lyophilized eluates of the hydroxylapatite column (Fraction I) was dissolved in a small amount of water and treated at room temperature for 5 to 10 minutes with Norit of a weight equal to that of the lyophilized preparation. The charcoal was centrifuged down and washed with a small aliquot of water. The supernatant solution and wash, which were combined, contained 96 per cent of the initial radioactivity. Four volumes of ethanol were added to the solution. From this step on, care was taken to protect the preparation from light. The mixture was heated at 72 to 74°C for 20 minutes and centrifuged. The denatured protein residue was treated with another four volumes of 80% ethanol and heated at 78°C for 10 minutes. The mixture

was centrifuged and the supernatant solution was combined with the first ethanol extract. A total of 96 per cent of the radioactivity was extracted into the ethanol solution. The ethanol extract was evaporated to dryness under reduced pressure at 40°C. The pink-colored residue was taken up in a small aliquot of water and its absorption spectrum was taken (Fig. 1, Curve B). The solution was again evaporated to dryness and the residue was transferred onto a sheet of Whatman No. 52 filter paper. Vitamin B₁₂ was also spotted as a control. On electrophoresis for 3 hours in 0.025 *M* KPO₄ (1:1) buffer at 2 kilovolts over a distance of 56 cm., a pink band was observed to move towards the anode and was separated by 5.3 cm. from vitamin B₁₂ which is electrophoretically neutral at pH 7. The pink band was cut out and eluted with 0.5 ml. of water. Sixty-nine per cent of the radioactivity in the ethanol extract was recovered in this single band. No other visible bands were detected. The spectrum of the eluate from the pink band is shown in Fig. 1 (Curve C).

Re-electrophoresis of such a preparation under the same conditions resulted in a single visible band which moved approximately the same distance towards the anode. Upon elution 52 per cent of the radioactivity applied to the second electrophoresis was recovered. The loss has not been accounted for. The spectrum of the pink band after the second electrophoresis is shown in Fig. 1 (Curve D). The main difference in spectra of the material before and after the second electrophoresis resides in the ultraviolet region. Since the material applied for the second electrophoresis was small in quantity, the accuracy of the absorbancy measurements was less satisfactory in the ultraviolet than in the visible region. The filter paper (Whatman No. 52) contains some impurities which absorb ultraviolet light with a maximum at 260 mμ. In order to correct for this background absorption, approximately the same area as the area of the pink band was cut out from the same sheet and the spectrum of the paper blank was subtracted from

the observed spectrum of the sample. This correction could result in considerable error in the estimation of a real spectrum of the compound when the amount of the material is small.

For comparison, the spectrum of DBC coenzyme was reproduced from the paper by Barker *et al.* in Fig. 5 (9). The spectrum

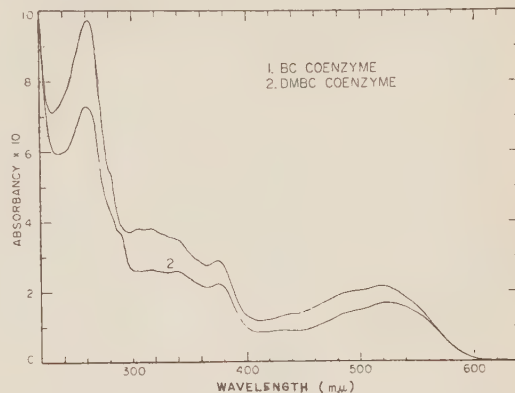


Fig. 5. Absorption spectra of 2.80×10^{-5} *M* BC coenzyme and 2.10×10^{-5} *M* DMBC (DBC) coenzyme in 0.01 *M* potassium phosphate buffer, pH 6.8. (Reproduced from the paper by Barker *et al.* (9)).

of B₁₂ coenzyme M has an absorption characteristic intermediate between those of vitamin B₁₂ and DBC coenzyme. B₁₂ coenzyme M has a broad absorption band with two plateaus at 510 mμ and 540 mμ. Vitamin B₁₂ and DBC coenzyme have a comparable broad band, but that of Vitamin B₁₂ is situated towards longer wavelengths, while that of DBC coenzyme is found at shorter wavelengths. In the case of the B₁₂ coenzyme M, absorption at the lower wavelength (510 mμ) is higher than that at the higher wavelength (540 mμ), but in the case of vitamin B₁₂ and DBC coenzyme this relationship is reversed. The conspicuous absorption plateau observed at 470 mμ in the spectrum of B₁₂ enzyme has completely disappeared. B₁₂ coenzyme M has a sharp peak at 364 mμ. The ratio of the intensity of the peak at 364 mμ to that of the broad band at the 500 mμ region is lower than that of vitamin B₁₂, but higher than that of DBC coenzyme. The

position of the peak is between those of vitamin B₁₂ and DBC coenzyme. Unequivocal description of the absorption pattern in the ultraviolet region may have to await further purification. The relative absorption intensity at 260 m μ of B₁₂ coenzyme M is much lower than that of DBC coenzyme, but considerably higher than that of vitamin B₁₂. The position of the peak is approximately 277 m μ , but there is also an absorption plateau at 260 m μ .

Preliminary studies were undertaken to determine the effect of various physical and chemical treatments on the spectrum of B₁₂ coenzyme M. A solution of B₁₂ coenzyme M was irradiated with aeration for two hours under two 15 watt fluorescent lamps at a distance of 15 cm. at room temperature. The resulting spectrum is shown in Fig. 6. The peak at 364 m μ was shifted to a shorter wavelength (353 m μ) with an increase of optical density. The appearance of a high peak at this wavelength was also reported to occur when AC coenzyme was irradiated (17). When 0.1 M KCN was added to the irradiated and aerated B₁₂ coenzyme M solution, the solution turned purple and the spectrum taken showed characteristic peaks of dicyanocobalamin. When this solution was acidified with HCl, the spectral change resembled that obtained when dicyanocobalamin is converted to monocyancobalamin (vitamin B₁₂) by acidification (Fig. 6).

Although the quantity and purity of the coenzyme solution used in this experiment were not satisfactory, the molar extinction coefficient (ϵ) at 364 m μ of B₁₂ coenzyme M was calculated to be 18.8×10^3 by assuming that the molar extinction coefficient of the peak at 366 m μ of the product formed by addition of KCN to the coenzyme solution is the same as that of the corresponding peak of dicyanocobalamin (molar extinction coefficient, 34.4×10^3 (18)). Molar concentrations of B₁₂ coenzyme M appearing in the legends for Fig. 1, 2 and 6 and in Table II were tentatively calculated by using this value.

Comparison of the Ability of B₁₂ Coenzyme M and Vitamin B₁₂ to React with Apoenzyme to

Yield B₁₂ Enzyme.—The activity of B₁₂ coenzyme M as a substrate for the formation of B₁₂ enzyme from the apoenzyme has been measured according to the procedures described previously (3), and is compared with the activity of vitamin B₁₂ (Table II).

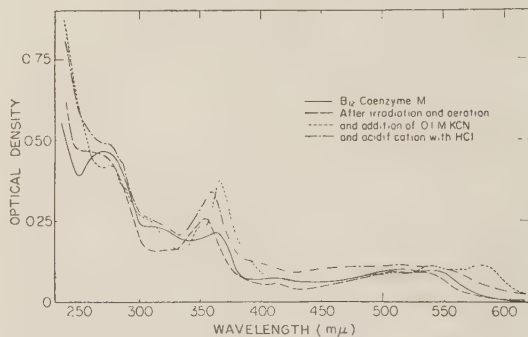


FIG. 6. Transformation of the spectrum of B₁₂ coenzyme M by exposure to light, addition of potassium cyanide and acidification. The coenzyme solution (approximately 1.1×10^{-2} mM) used in this experiment was obtained from a B₁₂ enzyme preparation which was purified by the procedures described in one of the previous papers (12). See the text for experimental details.

B₁₂ coenzyme M is more active than vitamin B₁₂ when the reaction mixture contained only apoenzyme and homocysteine in addition to the cobamide compounds tested. However, addition of the components required for the optimal formation of B₁₂ enzyme from the apoenzyme and vitamin B₁₂ also increases the formation of B₁₂ enzyme from apoenzyme and B₁₂ coenzyme M. In the case of vitamin B₁₂, omission of folate-H₄ or FAD impairs the activity of vitamin B₁₂ markedly, although the effect of omission of ATP was much less conspicuous than in the experiment previously reported (3). On the other hand, in the case of B₁₂ coenzyme M, the addition of two of the three compounds (folate-H₄, FAD, and ATP+Mg⁺⁺) resulted in the formation of B₁₂ enzyme to approximately the same extent as that found under optimal conditions (Table II, Experiment A and B). At a concentration of about 10^{-3} mM a greater amount of B₁₂ enzyme activity is found with vitamin B₁₂ as substrate than

TABLE II

Activity of B₁₂ Coenzyme M and Vitamin B₁₂ in Formation of B₁₂ Enzyme from Apoenzyme

Incubations were carried out under the conditions described in one of the previous papers (Table III in reference (3)), unless otherwise noted.

Omission in preliminary incubation	Methionine synthesized (mμmole/ml.)					
	Experiment A				Experiment B	
	Vitamin B ₁₂		B ₁₂ coenzyme M		Vitamin B ₁₂	B ₁₂ coenzyme M
	8.0×10 ⁻⁴ mM	8.0×10 ⁻⁵ mM	8.4×10 ⁻⁴ mM	8.4×10 ⁻⁵ mM	8.0×10 ⁻⁴ mM	7.0×10 ⁻⁴ mM
None (control) ^{a)}	13				26	28
None		29		65		
None	130		84		105	70
None ^{b)}			56			
None ^{c)}					128	59
Folate-H ₄	41		86		50	66
FAD	82		88		54	70
ATP, MgSO ₄	117		86		94	61
Folate-H ₄ , FAD					26	43
FAD, ATP, MgSO ₄					56	56
Folate-H ₄ , ATP, MgSO ₄					56	50
Folate-H ₄ , FAD, ATP, MgSO ₄	25		55		24	49

a) Cobamide compounds were omitted during the preliminary incubation, but added in the final incubation.

b) 10⁻² mM of antimetabolite of B₁₂, ethylamide derivative of B₁₂, was added in the preliminary incubation.

c) Cobamide compounds were incubated for 1 hour under nitrogen with homocysteine and FAD in KPO₄ (4:1) buffer prior to the preliminary incubation in the absence of apoenzyme. The concentration of each component during the non-enzymatic incubation was five times the standard concentration in the preliminary incubation mixture.

with B₁₂ coenzyme M. However, when comparison is made at about 10⁻⁴ mM, the relationship is reversed (Table II, Experiment A). The antimetabolite, ethylamide derivative of vitamin B₁₂, inhibits the formation of B₁₂ enzyme from vitamin B₁₂ as much as 80 per cent, when the former is present at ten times the concentration of vitamin B₁₂ (3). Under the comparable circumstances, the inhibition of B₁₂ enzyme formation from B₁₂ coenzyme M is much less (40 per cent) than from vitamin B₁₂.

DISCUSSION

The spectrum of B₁₂ enzyme will be an important clue for understanding the state

or structure of the cobamide prosthetic group *in situ* on the enzyme. The similarity of the absorption in the visible region of the spectrum between B₁₂ enzyme and vitamin B_{12r} suggests that cobalt in B₁₂ enzyme is divalent. The non-enzymatic reaction observed between vitamin B₁₂ and FAD in the presence of homocysteine and effect of FAD on the formation of B₁₂ enzyme support the above hypothesis. Furthermore, when vitamin B₁₂ was allowed to react with homocysteine and FAD prior to the addition of apoenzyme, a significant increase was observed in the formation of B₁₂ enzyme over the B₁₂ enzyme activity produced in the vessel in which the non-enzymatic incubation did not

precede the "preliminary incubation" (Table II, Experiment B). This observation also suggests the involvement of the non-enzymatic reaction, presumably reduction, of vitamin B₁₂ with homocysteine and FAD in the formation of B₁₂ enzyme from vitamin B₁₂.

On the other hand, the catalytic effect of vitamin B₁₂ on reduction of FAD might be of some significance in view of the fact that FADH₂ is involved in the enzymatic synthesis of the methyl group of methionine (12).

When the cobamide coenzyme is removed from the protein moiety of B₁₂ enzyme, a rather drastic change in its spectrum occurs. The interpretation of the spectral change is difficult at this time. The removal of protein may cause the spectral change without conversion of cobalt valency. On the other hand it is also possible that the divalent cobalt is protected from the oxidation by a protein ligand, but is quickly oxidized upon dissociation. The effect of homocysteine and FAD on the spectrum of B₁₂ coenzyme M has not been studied.

The effect of light and aeration, addition of KCN, and subsequent acidification more or less simulated the effects of these treatments on the cobamide coenzymes of Barker *et al.* (17). Since the preparation used in the experiment was not satisfactory both in quantity and purity, precise analyses have not been possible. Another important observation is that B₁₂ coenzyme M is electro-negative even at the pH (approximately 2.5) of 0.5M acetic acid. This characteristic is unique among cobamide derivatives so far reported.

SUMMARY

1. The B₁₂ enzyme was partially purified from 1 kg. of cells of *Escherichia coli* mutant 113-3 grown on vitamin B₁₂ and its absorption spectrum was taken. The spectrum of B₁₂ enzyme showed an absorption characteristic similar to that of cobaltous cobamide derivatives.

2. Spectral changes of both vitamin B₁₂ and FAD were found to be accelerated by

each other when the two compounds were mixed with homocysteine in a phosphate buffer at pH 7.5. The implications of these findings in relation to the enzymatic system for methionine synthesis were discussed.

3. An unknown cobamide derivative was released from the purified B₁₂ enzyme. Spectral and electrophoretic studies were made of the released material. On electrophoresis in 0.5M acetic acid, the cobamide derivative moved towards the anode.

REFERENCES

- (1) Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Peterson, A., Toohey, J. I., Ladd, J. N., Volcani, B. E., and Wilson, R. M., *J. Biol. Chem.*, **235**, 181 (1960)
- (2) Kisluk, R. L., and Woods, D. D., *Biochem. J.*, **75**, 467 (1960)
- (3) Takeyama, S., Hatch, F. T., and Buchanan, J. M., *J. Biol. Chem.*, **236**, 1102 (1961)
- (4) Eggerer, H., Stadtman, E. R., Overath, P., and Lynen, F., *Biochem. Z.*, **333**, 1 (1960)
- (5) Lengyel, P., Mazumder, R., and Ochoa, S., *Proc. Nat'l Acad. Sci. U. S.*, **46**, 1312 (1960)
- (6) Abeles, R. H., and Lee, H. A., Jr., *J. Biol. Chem.*, **236**, PC 1 (1961)
- (7) Barker, H. A., Weissbach, H., and Smyth, R. D., *Proc. Nat'l Acad. Sci. U. S.*, **44**, 1093 (1958)
- (8) Weissbach, H., Toohey, J. I., and Barker, H. A., *Proc. Nat'l Acad. Sci. U. S.*, **45**, 521 (1959)
- (9) Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N., and Volcani, B. E., *J. Biol. Chem.*, **235**, 480 (1960)
- (10) Guest, J. R., Helleiner, C. W., Cross, M. J., and Woods, D. D., *Biochem. J.*, **76**, 396 (1960)
- (11) Guest, J. R., *Biochem. J.*, **76**, 405 (1960)
- (12) Hatch, F. T., Larrabee, A. R., Cathou, R. E., and Buchanan, J. M., *J. Biol. Chem.*, **236**, 1095 (1961)
- (13) Diehl, H., and Murie, R. *Iowa State Coll. J. Sci.*, **26**, 555 (1952)
- (14) Beaven, G. H., and Johnson, E. A., *Nature*, **176**, 1264 (1955)
- (15) Johnson, A. W., and Shaw, N., *Proceedings of Chem. Soc.*, 421 (1960)
- (16) Smith, E. L., "Vitamin B₁₂." Methuen and Company, Ltd., London p. 50 (1960)
- (17) Weissbach, H., Ladd, J. N., Volcani, B. E., Smyth, R. D., and Barker, H. A., *J. Biol. Chem.*, **235**, 1462 (1960)
- (18) George, P., Irvine, D. H., and Glauser, S. C., *Annal. New York Acad. Sci.*, **88**, 393 (1960)

On the Minimum Requirement and Treatment Dosis
of Thiamine for Rats

By NAGAO SHIBATA, SEIZO YAMASHITA and KAZUTO YASUDA

(From the First Department of Internal Medicine, Faculty of Medicine, University
of Tokyo, Tokyo)

(Received for publication, February 28, 1961)

When vitamins are given to animals in excess, a great amount is decomposed or excreted, and only a very small portion is utilized by the body. But when they are given in small amounts, the body is very careful in utilizing them and does not waste them. Even though some vitamins are completely excluded from the diets, the rats do not show deficiency symptoms of the vitamins, as these are manufactured in their intestines by bacteria and absorbed and utilized within their bodies. The purpose of this work is to find out the effect of thiamine given in very small amounts orally to rats.

METHODS

1. *Feedings*—Young small rats strain were kept at first on normal diets. When their body weight became about 50 to 60 g., they were divided into 6 groups, the first a control normal group, the second a thiamine deficient group, the third a group to which 2.5 μ g. of thiamine was given every day, the fourth a group to

which 2.5 μ g. of thiamine was given in the fourth or fifth week of thiamine deficiency, the fifth a group to which 5 μ g. of thiamine was given in the fourth or fifth week of thiamine deficiency. The diet and the method of feeding are given in Table I.

2. *Determination of Thiamine*—The thiamine contents in the organs and in the gastrointestinal tracts of rats were measured by the fluorometric method of Fujiwara (1).

3. *Electrocardiographic Examination*—Unanesthetized rats were placed (2) on their abdomen on a board to which their feet were tied, and electrocardiographic recording were made. Electrodes were dipped in the electrode paste and these were taped to the feet soles of the rats with adhesive plaster. The faces of the rats were covered with gauze mask to keep them still. Electrocardiographic examination was started several minutes after the rats became quiet and standard limb and unipolar limb lead were recorded. The standardization used was the same as in men's electrocardiography (1 mvolt:1 cm.). The room temperature was kept at about 20–25°C at all times during the examination.

TABLE I
Composition of Diet for Rat

Basal diet		Salt mixture ¹⁾		Vitamin B ₂ group	
Casein	20%	NaCl	20.2	Riboflavin	40 μ g.
Starch	68	MgSO ₄ ·7H ₂ O	63.5	Pyridoxine	50
Salt mixture ¹⁾	4	K ₂ HPO ₄	111.5	Nicotinnic acid	200
Soy-bean oil	5.5	Ca-lactate	28.7	Ca-Pantothenate	200
Cod liver oil	2.5	Na ₂ HPO ₄ ·12H ₂ O	52.6	Choline-HCl	900
Water		Ca ₂ H ₂ (PO ₄) ₂ ·H ₂ O	111.6	Inositol	180
		Fe-citrate	13.8	B ₁₂	0.024
These are cooked and made into cakes.				The above are made into 0.1 ml. of suspension.	

1) The basal diet is given in a larger amount than that rats can eat. B₂ group suspension (0.1 ml.) is given orally daily. B₁ is given in various doses (or not given) orally, according to experimental conditions.

RESULTS

1. *Thiamine Deficient Group*—In the first week of thiamine deficiency, growth, appetite, vitality and stool of this group were the same as those of the normal group. From the middle of the second week, the appetite decreased gradually to the daily intake of about 15–20 g. of the diet, and the growth stopped, but vitality and stools were still good. In the third week appetite decreased to the daily intake of about 10–15 g. of the diet, that is, about one-half of the intake of normal control rats, and the weight loss appeared clearly. But in general there was no change in the vitality and character of the stools. In the fourth week the appetite decreased greatly and the daily food intake became only about 10 g. The body weight decreased markedly and showed a daily average loss of several grams. Most of rats lost their vitality and revealed paralytic symptom (in walking) and got diarrheas. Some rats died at the end of this week. In the fifth week the rats lost their appetite almost completely and daily food intake was only about 5 g. The body weight decreased extremely. Marked neuro-paralytic symptoms and ataxia were observed during this period. All the rats became enervated and had stinking black-brown diarrhea. Most of rats died during this week (Fig. 1). When the rats in the fourth or fifth week of thiamine deficiency were kept in cages without taking away their feces, most of the rats

showed the fece in their stomachs, moreover, at times the fece was found even in the duodenum. In this stage, about 0.3 μ g. of thiamine was found in the stomach and about 5 μ g. of thiamine could always be found in the cecum. Furthermore, even though about

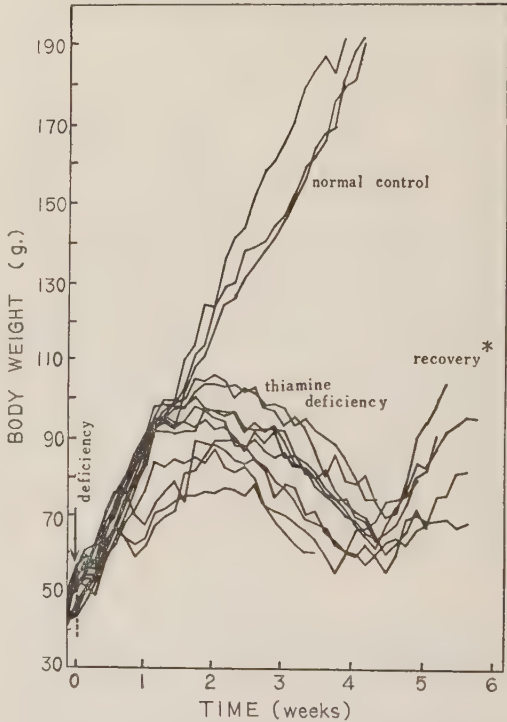


FIG. 1. The progress of body weight of control normal rats and thiamine deficient rats.
* by the administration of thiamine (5 μ g.) orally daily.

TABLE II
Thiamine Content in the Gastrointestinal Tract of Rats
(the average of thiamine content of 3 cases)

Condition	Stomach	Small intestine	Large intestine	Total
	μ g.	μ g.	μ g.	μ g.
B ₁ deficiency group	0.25 (—)	0.46 (0.16)	5.39 (0.54)	6.10 (0.70)
5 μ g. of B ₁ (orally daily) group	0.29 (0.08)	1.18 (0.38)	5.97 (0.71)	7.44 (1.17)
10 μ g. " (" ") "	0.47 (0.10)	1.48 (0.48)	6.92 (1.24)	8.87 (1.82)
40 μ g. " (" ") "	0.52 (0.10)	4.31 (0.60)	8.61 (1.86)	13.44 (2.56)

Determination of the thiamine contents was carried out 24 hours after the administration of thiamine.

The figures in the parenthesis show the free thiamine content.

5–6 $\mu\text{g.}$ of thiamine could always be found in the gastrointestinal tract from the first stage of thiamine deficiency to the time just before the death, the rats lost their body weight and became enervated. And in this stage the ECG of rats showed various marked changes, such as bradycardia, arrhythmia (sinus arrhythmia, a-v block, interpolated nodal rhythm, and premature beat), increase of right axis deviation, ST changes (elevation and depression) and T changes (elevation and depression or negative) *etc.* (2).

2. *Cases to Which 2.5 $\mu\text{g.}$ of Thiamine Was Given Orally Daily*—When the rats were given 2.5 $\mu\text{g.}$ of thiamine orally daily, they grew rapidly. They gained 3–5 g. daily, but stopped growing when their body weight reached 80–90 g. (Fig. 6), but their general conditions were good. The amount of thiamine in the gastrointestinal tract was 5–6 $\mu\text{g.}$, the same amount as in all stage of thiamine deficiency. The amount of thiamine in the principal organs of the rats was very little and it was found that the amount was the same as in the early stage of thiamine deficiency. There was no change in ECG. Judging from the above it is assumed that the minimum oral requirement of thiamine in rats to maintain the body weight at 70–80 g. is 2.5 $\mu\text{g.}$

In this group it can be assumed that the thiamine was completely absorbed and utilized by the body.

3. *Cases to Which the 2.5 $\mu\text{g.}$ of Thiamine*

Was Given Orally in the Fourth or Fifth Week of Thiamine Deficiency—When 2.5 $\mu\text{g.}$ of thiamine was given orally to thiamine deficient rats, the rats regained vitality after 24 hours, but in this case their body weight decreased. The ECG abnormalities observed on the previous day improved to some extent (Fig. 2, 3, 4).

However, after 48 hours they returned to their former state (Fig. 5). There was no change in the amount of thiamine in the principal organs compared with the thiamine deficient rats.

4. *Cases to Which 5 $\mu\text{g.}$ of Thiamine Was Given Daily Orally*—When 5 $\mu\text{g.}$ of thiamine was orally given daily the rats kept growing evenly to the body weight of 140–160 g. (Fig. 6). Their general conditions were very good, they didn't eat their feces, and there was no change in ECG. On the other hand the amount of thiamine in the gastrointestinal tract was the same as in the thiamine deficient rats. There was no histological change in the principal organs.

5. *Cases to Which 5 $\mu\text{g.}$ of Thiamine Was Given Orally in the Fourth or Fifth Week of Thiamine Deficiency*—When thiamine deficient rats in the fourth or fifth week were given 5 $\mu\text{g.}$ of thiamine orally, they regained their vitality and gained weight after 24 hours. The ECG abnormalities observed on the previous day improved greatly, but the amount of thiamine in the principal organs showed no change, compared with the thiamine de-

TABLE III
Thiamine Content of the Organs of Rats

Organs	thiamine deficient rats (in the 4th week of deficiency) (average of 3 cases)		the rats given 5 $\mu\text{g.}$ of thiamine orally daily (average of 4 cases)		Control normal rats given 40 $\mu\text{g.}$ of thiamine orally daily			
					[I] (average of 4 cases)		[II] (average of 4 cases)	
	$\mu\text{g.}$ 6.9	$\mu\text{g.}$	$\mu\text{g.}$ 7.7	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$ 26.7	$\mu\text{g.}$
blood								
liver	30.9 (3.1)	1.15 (0.06)	92.2 (5.6)	6.51 (0.40)	513(48)	12.4 (1.3)	311 (24)	13.8 (1.1)
kidney	50.0 (4.7)	0.50 (0.05)	61.8 (18.5)	1.05 (0.31)	334	2.02	196 (15)	1.82(0.1)
heart	22.3 (3.9)	0.11 (0.04)	57.5 (2.1)	0.30 (0.01)	682	1.26	376	1.61
brain	62.5 (7.4)	0.87 (0.19)	100.3 (4.9)	1.59 (0.08)	320	4.58	269	3.83
muscle	16.0 (3.4)		18.4 (3.7)		171		102	

The figures in parenthesis show free thiamine contents.

The average of body weights of rats—thiamine deficient group, control group (I): 47 g., 5 $\mu\text{g.}$ of thiamine group, control group (II): 150 g.

ficient rats. They did not eat their feces. When 5 μ g. of thiamine was administered every day, paralytic symptoms and diarrhea were cured within several days, and general state thereafter was similar to that of the normal group.

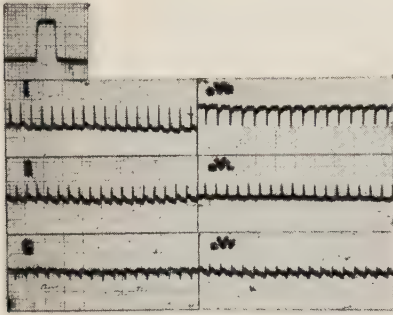


FIG. 2. Electrocardiogram of a normal rat (body weight 179.5g). Heart rate 530/min.

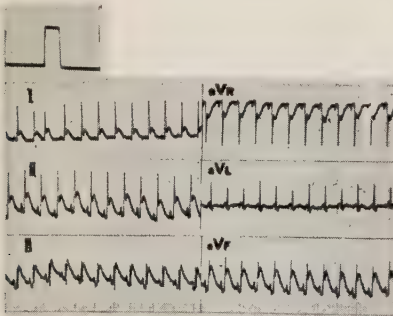


FIG. 3. Electrocardiogram of a rat on the 20th day of thiamine deficiency. Arrhythmia (sinus arrhythmia and ventricular premature beat) and bradycardia were observed. Heart rate 300~375/min.

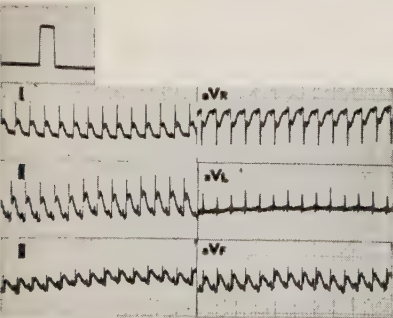


FIG. 4. One day after oral administration of 2.5 μ g. of thiamine. Arrhythmia disappeared and bradycardia improved. Heart rate 409/min.

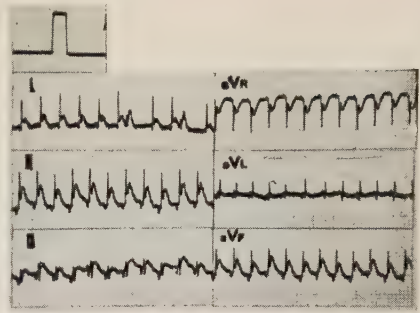


FIG. 5. Two days after oral administration of 2.5 of thiamine. Conditions took a bad turn—arrhythmia (sinus arrhythmia) appeared and heart rate fell. Heart rate 346~375/min.

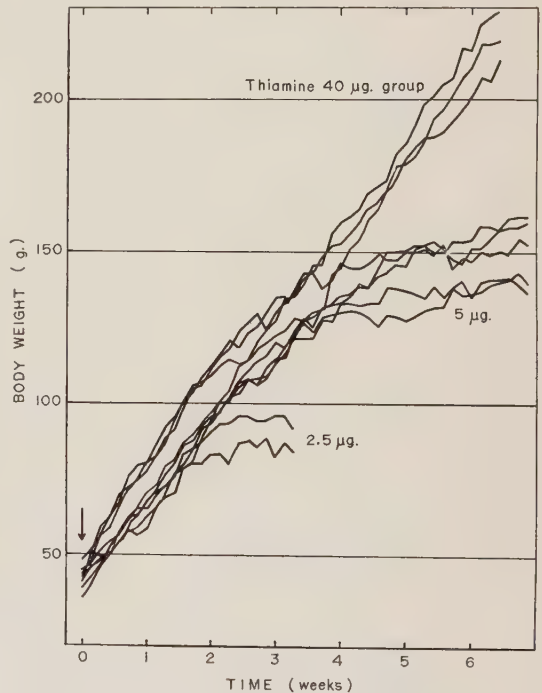


FIG. 6. The progress of body weight of rats administered in a daily doses of (2.5, 5 or 40 μ g.) of thiamine (orally).

The basal diet is given in a larger amount than that the rats can eat. B₂ group is given orally daily.

DISCUSSION

Many studies hitherto have been made on the requirement of thiamine of the rats by Cowgill, *et al.* and many others, but few reports are found which deal with the

thiamine content in the gastrointestinal tracts of rats (3-6). In the gastrointestinal tracts of thiamine deficient rats thiamine was always found and about 5 μ g. of thiamine was found in the caecum from the early stage of deficiency until death. On the other hand after the third week of deficiency the body weight decreased steadily and they lost their vitality. At the fourth week and fifth week, the body weight fell down to the level of the beginning of deficiency and the rats died. But the rats which were given 2.5 μ g. of thiamine orally everyday grew rapidly up to about 80 g. but at this stage the increase in the body weight stopped, and thereafter there was no change in weight but they maintained their health. On the other hand, when the daily dosis of thiamine given orally was increased to 5 μ g., they gained weight rapidly to 140-160 g. Both of these groups showed no change in ECG. Judging from the above, with each 30 g. of increase of the body weight it is necessary to increase 1 μ g. of the dosis of thiamine.

In the cases when 2.5 μ g. of thiamine was given orally to rats in the first stage of thiamine deficiency, their appetite increased in 24 hours and their general conditions as well as ECG improved although their body weight decreased. The amount of thiamine in the gastrointestinal tract was almost the same as in the case of the thiamine deficient rats. And then, owing to the enhancement of catabolism the rats lost weight despite the increase of food intake. The amount of thiamine in the gastrointestinal tract was the same, whether thiamine was administered or not. It might suggest that the thiamine in the bacteria is not absorbed as such in the intestines. The fact that there was no difference in the amount of thiamine in the organs suggests that the thiamine absorbed by the rats became completely utilized within a day.

When 5 μ g. of thiamine was administered orally, the rats gained weight of about 5 g. and the ECG changes improved, but after 48 hours their conditions started to worsen again. However, when 5 μ g. of thiamine was given orally daily, the ECG changes become normal again within four or five days, and

the body weight increased daily. The amount of thiamine in the gastrointestinal tract of these rats was 5 μ g., and the amount of thiamine in their principal organs was slightly more than in the case of rats that were not given thiamine orally at all. Judging from this, it is assumed that 5 μ g. is the minimum amount as the curative dosis. The fact that acute human beriberi that was prevalent in the old days showed remarkable improvement with very little amount of thiamine has a very close resemblance to these findings on thiamine deficient rats.

SUMMARY

1. In the gastrointestinal tracts of thiamine deficient rats about 5 μ g. of thiamine is always found until their death, but it is assumed that this is not utilized by the rats.

2. When 2.5 μ g. of thiamine is administered orally daily, the rats grow up to about 80-90 g. and then stop growing. On the other hand, when 5 μ g. is administered orally daily, they grow up to about 150 g. and then stop growing. In both cases 5-6 μ g. of thiamine can be found in the gastrointestinal tracts, and the amount of thiamine in the vital organs is almost the same as when no thiamine is administered.

Daily administration of 2.5 μ g. of thiamine maintains the health condition of the rats up to about 80 g. of body weight, and daily administration of 5 μ g. thiamine maintains the health condition up to about 150 g.

3. The minimum amount of thiamine necessary for curative treatment of the rats in the fourth or fifth week of thiamine deficiency was found to be between 2.5 μ g. and 5 μ g.

The authors are deeply grateful for his encouragement to Prof. S. Tazaka, in whose Department this work was done. The authors are also grateful to Dr. T. Takagi for his helpful advice.

REFERENCES

- (1) Fujiwara, M., and Matsui, K., *Anal. Chem.*, **25**, 810 (1953)
- (2) Yoshitoshi, Y., Shibata, N., and Yamashita, M., *Japanese Heart Journal*, **2**, 42 (1961)

- (3) Cowgill and Robinson, "The Vitamine B Complex," Capman & Hall, p. 82 (1951)
- (4) Dann, M., and Cowgill, G. R., *Am. J. Physiol.*, **109**, 27 (1934)
- (5) Shibata, N., *Vitamins*, **3**, 78 (1950)
- (6) Emerson, G. R., *Proc. Soc. Exptl. Biol. Med.*, **59**, 299 (1945)

Some Properties of Muscle Glycerate 2, 3-Diphosphatase and Activation by Mercuric Ion*

By TAKASHI HASHIMOTO, MAKOTO NAKAO and HARUHISA YOSHIKAWA

(From the Department of Physiological Chemistry and Nutrition,
Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, February 28, 1961)

A specific phosphatase for D-2, 3-diphosphoglyceric acid (2, 3-DPGA) has been described by Rapoport and Luebering (1), and thereafter Joyce and Grisolia (2) have studied the properties of the purified enzyme obtained from chicken breast muscle and baker's yeast. While the enzyme preparations described by Rapoport *et al.* (1, 3) were markedly activated by mercuric ion, Joyce and Grisolia preparations were not activated by this ion. The latter investigators suggested two possibilities: 1. that mercuric ion would eliminate the inhibitor from the crude enzyme preparation or 2. that the enzymes from different tissues or species would show different properties.

The present communication deals with some properties of the enzyme preparations obtained from rabbit skeletal muscle, with special reference to the effect of mercuric ion.

EXPERIMENTALS

Materials—2, 3-DPGA was isolated from pig blood (5). DL-2-phosphoglyceric acid (2-PGA) was synthesized from β -glycerophosphate as described by Kiessling (6) and purified as described by Warburg and Christian (7). s-Collidine was purified *via* urea complex (8). Other materials were commercial products.

Methods—The solution of 2, 3-DPGA and other phosphorus compounds were freed from barium by decationization using cation exchanger (Amberlite IR-120-H⁺) and neutralized with NaOH.

Inorganic phosphate was determined by the

method of Fiske-Subbarow (9), mono- and di-phosphoglyceric acid by the chromotropic acid reaction of Bartlett (10), and protein by the method of Lowry *et al.* (11). Paper chromatographic separation of 3-phosphoglyceric acid (3-PGA) and 2-PGA was carried out according to the method of Cowgill (12) except that the authors used ascending technique for better separation.

Assay—In typical experiments, the following components were mixed in a final volume of 1.0 ml.: 3μ moles 2, 3-DPGA; 5μ moles s-collidine-HCl buffer at pH 7.0 and the enzyme preparation. After incubation, the reaction was stopped by the addition of 1.0 ml. of 0.6 *N* perchloric acid, followed by centrifugation and the aliquot of the supernatant was used for the determination.

Definitions of the enzyme activity unit and specific activity were given according to Rapoport and Luebering (1).

RESULTS

Purification—Purification of the enzyme was carried out according to Joyce and Grisolia (20) with a slight modification. Each gram of acetone powder of rabbit skeletal muscle was extracted with 10 ml. of ice-cold water (Crude extract). To each 1 ml. of the extract, 0.67 ml. of ethanol was added and after centrifugation at -10°C , 0.71 ml. of ethanol was slowly added to the supernatant and centrifuged. The precipitate thus obtained was dissolved in water (EtOH-Fr. 1 see Table 1).

A solution of mercuric acetate and 2, 3-DPGA in Tris buffer, pH 7.0, was added to the Fraction 1, as described by Joyce and Grisolia (2). The mixture was incubated and then chilled. The precipitate was discarded after centrifugation, and the supernatant was retained (Hg-Fr.). To each ml.

* This work was aided by a grant from the Scientific Research Fund of the Ministry of Education. A preliminary report was presented at the annual meeting of the Japanese Biochemical Society, in November, 1960.

TABLE I
Protocol of the Purification of the Enzyme

Fraction	Total volume ml.	Total Unit	Total Protein mg.	Specific Activity	Yield per cent
Crude Extract	360	572	2543	0.23	100
EtOH-Fr. 1.	138	521	762	0.68	92
Hg-Fr.	190	804	745	1.08	141
EtOH-Fr. 2.	57	370	87	4.25	65
IRC-50 Fr.	48	245	35	7.00	43
Final Fraction	46	241	28	8.53	42

50 g. of acetone powder was used, and each step of the purification and the definition of the enzyme activity are described in text.

of this fraction was added 0.43 ml. of ethanol and centrifuged and the precipitate was discarded. To each ml. of the supernatant obtained, 1 ml. of ethanol was added again. After centrifugation, the precipitate was dissolved in water (EtOH-Fr. 2).

The EtOH-Fr. 2 was run though an IRC-50 column which had been previously bufferized with 0.005 *M* sodium citrate (pH 6.0), and the resin was washed with the citrate buffer of twice the volume of the column. The percolates were combined (IRC-50 Fr.). The excess of mercuric ion in the enzyme preparation was removed by the alcohol precipitation and treatment with IRC-50. Any insoluble material found was discarded by centrifugation and the supernatant solution (Final Fr.) was stored at about -20°C . The enzyme at this stage was stable and could be kept in the deep-freeze for over one month without any loss of activity.

A summary of the results is shown in Table I.

Though Joyce and Grisolia (2) added ethanol to the enzyme preparations in the presence of 2, 3-DPGA, no advantage of such treatment observed in our case. Therefore it was used only at the mercuric-treatment stage. As a starting material, acetone powder and water extract of fresh muscle gave the same results.

Stoichiometry—As it was previously described, 2, 3-DPGA was hydrolyzed to inorganic phosphate and 3-PGA (1). The authors attempted to examine the same scheme.

Whereas Joyce and Grisolia have reported that the enzyme preparations from chicken breast muscle were not free from monophosphoglycerate mutase, the mutase activity was not detected in the purified preparation of rabbit muscle described here. To establish the stoichiometry of the enzyme reaction, aliquots of the mixture were deproteinized at various intervals, and the phosphorus compounds were precipitated as barium salts, and converted to free acid with the use of a cation exchanger. The components were separated by the method of Cowgill (12). During incubation 2, 3-DPGA was decreased and at the same time there was an increase in the amount of inorganic phosphate and 3-PGA, while no considerable amount of 2-PGA was formed. The results are presented in Table II.

TABLE II
Stoichiometry of the Glycerate 2,3-diphosphatase Reaction

Incubation Time (min.)	30	60	120
+ Δ Inorg. Phosphate (μ moles)	1.2	2.2	3.4
+ Δ 3-PGA (")	1.1	2.0	3.0
- Δ 2, 3-DPGA (")	1.3	2.3	3.0

Experimental procedures are shown in text.

Effect of Mercuric Ion—Activation of the enzyme by mercuric ion was reported by many investigators (1-4). The activity of the purified enzyme was slightly inhibited by KCN (10^{-3} – 10^{-2} *M*), and to about one-

third by saturation with H_2S . Although mercuric ion had an activating effect, the addition of a large amount of the ion (more than $10^{-3} M$) was inhibitory. Hydrogen sulfide restored the activity affected by excess mercuric ion at any stage of purification. Chelating agents such as ethylenediamine tetraacetic acid (EDTA), *o*-phenanthroline, α , α' -dipyridyl and dithizone showed a marked inhibitory effect on the purified enzyme. The attempt to eliminate mercuric ion from the enzyme by dialysis against the solution of EDTA was failed resulting in the precipitation of protein with the irreversible loss of activity. But when the dialysis was conducted against *s*-collidine-HCl buffer (4), no precipitation occurred. This treatment caused a decrease in specific activity to about one-tenth (longer the time of dialysis, the greater the decrease in specific activity). Among various metal ions so far tested (Mg^{++} , Ca^{++} , Co^{++} , Fe^{++} , Zn^{++} , Cu^{++} , 10^{-4} – $10^{-3} M$ and Hg^{++} and Ag^+), only Hg^{++} and Ag^+ were effective in reactivating the dialyzed enzyme. The effective concentration of Ag^+ was relatively high, and the precipitant of silver chloride was brought about in the reaction mixture. The addition of the optimum amount of mercuric ion to the dialyzed preparation increased the specific activity

about 8 to 10-fold; it was nearly equal in activity to the non-dialyzed enzyme.

To determine the mercury content of the enzyme preparation, radioactive mercury Hg^{203} was used in the mercuric-treatment procedure. Table III shows the relation of the specific activity and the mercury content at the various stages of preparation.

It has been shown that the repeated ethanol fractionation increase the specific activity and the dialysis against collidine buffer removed the bound mercuric ion with a profound loss of enzymatic activity. With these procedures, the ratios of the activity to the bound mercuric ion were constant in spite of the variation of the specific activity.

Effect of Salts—The high concentration of NaCl, KCl, NH_4Cl and Na_2SO_4 caused a decrease in activity (Table IV). The inhibitory effect of NaCl was competitive with 2, 3-DPGA and the effects of NaCl and EDTA were apparently additive. NaCl was mixed with the radioactive mercury bound enzyme solution to a final concentration of 0.5 *M*, and incubated at 37°C for 30 minutes. The protein was then separated from the salts with the Gel-filtration method (13). The chromatogram in this procedure is shown in Fig. 1.

TABLE III

The Relation of the Activity and Protein-Bound-Mercury

Fraction ¹⁾	Specific Activity	μ mole $\text{Hg}^{2)}$	Activity
		mg. Protein	μ mole Hg
EtOH-Fr. 1	0.64		
Hg-Fr.	1.44	0.071	20.3
EtOH-Fr. 2	2.25	0.094	23.9
EtOH-Fr. 3	5.3	0.32	16.6
Dialyzed Fr. 1	0.1	0.008	12.5
Dialyzed Fr. 2	0.6	0.054	11.1

1) Some procedures are shown in text. EtOH-Fr2 was refractionated with ethanol. The obtained fraction (EtOH-Fr. 3) was dialyzed against *s*-collidine-HCl buffer for 6 hours. (Dialyzed Fr. 2) or 20 hours. (Dialyzed Fr. 1).

2) Contents of mercury was determined with use of its radioactivity.

TABLE IV

Effects of Various Salts on the Enzyme Activity

Concentration of Salts (M)	NaCl	KCl	NH ₄ Cl	Na ₂ SO ₄
0	100	100	100	100
0.1	74	58	59	22
0.2	45	26	17	8
0.3	23	9	7	4
0.4	14	7	5	2
0.5	13	5	4	1

All activity was expressed as % activity of the control value.

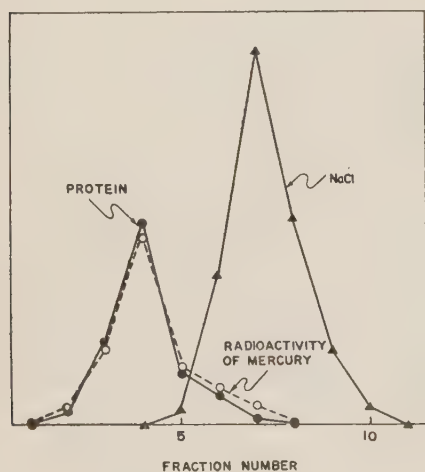


FIG. 1. Separation of the enzyme from salt with gel-filtration method.

Sodium chloride was added to the enzyme solution (Final Fr.) labelled with radioactive mercury up to the concentration of 0.5 M and incubated for 30 minutes at 37°C. The reaction mixture was run through the column of Sephadex G-25 (1×6 cm.) previously washed with distilled water. The effluent was fractionated by 1 ml. Each components is expressed by arbitrary unit.

Enzyme protein (—●—) is separated from salt (—▲—) and the radioactivity of mercury (...○...) is detected in the protein fraction.

Gel-filtration has been used for the rapid separation of the solutes by its size. The chromatogram in Fig. 1 shows that the radioactivity of mercury is present not in the salt fraction, but only in the protein fraction. The result indicates that the bound mercury cannot be eliminated from the enzyme protein

by the salt.

Inhibitor in Erythrocytes—It has been noted by us that hemolysates obtained from human, rabbit, cow and horse erythrocytes exhibit an inhibiting action on glycerate 2, 3-diphosphatase. Further studies revealed that IRC-50 treated hemoglobin fraction from these sources as well as horse crystalline hemoglobin showed the same inhibitory effect on the enzyme which had been previously treated with mercuric ion (see Table V). However, the inhibition was no longer observed, when the excess of mercuric ion was added to the reaction mixture, as indicated in Table V. Furthermore, the following experiment was

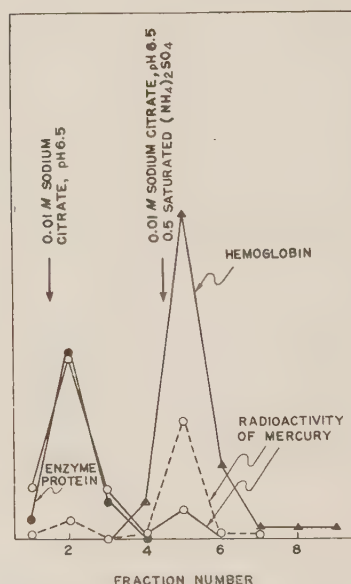


FIG. 2. Separation of the enzyme protein from added hemoglobin

The radioactive final enzyme fraction was mixed with horse crystalline hemoglobin (final concentration; 10^{-4} M), or not. The enzyme and the hemoglobin was separated by the column of IRC-50 (1×1.5 cm.) (see Experimental Method). The enzyme was eluted in the first fraction, and the hemoglobin was eluted with bufferized 0.5 saturated ammonium sulfate solution. The hemoglobin was eluted only 50 per cent and the residual was precipitated in the column. The precipitation was not occurred by salting-out.

—●—, Protein; —▲— Hemoglobin; ...○... Radioactivity in the presence of, and —○— in the absence of hemoglobin.

TABLE V
Effect of the Inhibitor Obtained from Erythrocyte
(A) Effect on the Mercury-Treated Fraction

Inhibitor or Mercuric Ion Added (M)			Relative Activity
Hemolysate ¹⁾	IRC-50 Treated ¹⁾ Hemoglobin Fraction	Mercuric Acetate	
0	0	0	100
2×10^{-4}	0	0	26
0	3×10^{-4}	0	21
2×10^{-4}	0	5×10^{-3}	66
2×10^{-4}	0	10^{-3}	153
2×10^{-4}	3×10^{-4}	5×10^{-4}	156

1) Concentrations of hemolysate and IRC-50 treated hemoglobin are expressed as hemoglobin.

(B) Effect on the Dialyzed Fraction (see Table III)

Hemoglobin or Mercuric Ion Added (M)		Relative Activity
Crystalline Horse Hemoglobin	Mercuric Acetate	
0	0	100
0	10^{-4}	503
10^{-4}	10^{-4}	66
10^{-4}	10^{-3}	240

conducted to elucidate the mechanism of inhibition. Horse crystalline hemoglobin was added to the enzyme preparation previously treated with isotopic mercury, and chromatographed on ion exchanger resin column. As indicated in Fig. 2, the radioactivity was transferred to the hemoglobin. These results indicated the inhibitor in erythrocytes was hemoglobin, by which mercuric ion was removed from the enzyme.

Effect of Thiols—The predialyzed fraction was not affected by mercaptoethanol, cysteine, lipoic acid or 2, 3-dimercapto-1-propanol ($10^{-3}M$). However, dialyzed enzyme was activated two-fold by thiols or serum albumin.

DISCUSSION

The phosphatase activity contained in fresh muscle of rat or rabbit was estimated with use of its homogenates. The activities obtained by us were nearly equal to that reported by Sutherland *et al.* (14), Rapoport and Luebering (1), but less than that

from chicken breast muscle (2). These data suggest that the activities are different with species.

According to Mányai and Varady (15), the erythrocyte phosphatase showed much smaller activity than the muscle phosphatase, and it was activated by sodium bisulfite and high concentrations of salts, but inhibited by mercuric ion. These facts were also confirmed in this laboratory except that the activation effects were rather less than those reported by Mányai and Varady. In the case of the muscle enzyme, however, these reagents showed opposite effects, namely, sodium bisulfite and high concentration of salts inhibited the enzyme activity, but mercuric ion activated it. These phenomena may suggest that the muscle phosphatase is different from the erythrocyte phosphatase.

The role of mercuric ion would be due to 1) the removal of the inhibitors or 2) the formation of an essential component of the enzyme. If the hypothetical inhibitor is

present in the enzyme preparation, it might be a large molecule substance (large molecule peptide or protein) as inferred from its non-dialysability. And it must be firmly bound to the mercuric ion relatively, because no effects of thiols or little effect of KCN on the mercuric ion treated enzyme was observed.

Even after the purification of the protein was carried out through mercuric ion treatment, followed by ethanol fractionations and IRC-50 treatment, the activity of the enzyme was reduced by demercurization procedures such as dialysis against s-collidine-HCl buffer or H₂S treatment. Moreover, the activity was restored by the addition of an optimum amount of mercuric ion. As shown in Table III, the ratio of the specific activity to the amount of mercury content was constant during some procedures.

The optimum amount of mercury bound to our enzyme preparation was 0.3 to 0.5 μ mole per mg. enzyme protein. This value is nearly equal to the amount of mercury of Joyce and Grisolia preparation.

The results of our experiment may indicate that mercury is essential to the activity of the enzyme.

An attempt to examine the possibility that the mercury plays a role really *in vivo*, is now in progress, though there remains another possibility that the phosphatase is denatured by the mercuric ion treatment and the obtained purified enzyme is an "artificial" one.

REFERENCES

- (1) Rapoport, S., and Luebering, J., *J. Biol. Chem.*, **189**, 683 (1951)
- (2) Joyce, B. K., and Grisolia, S., *J. Biol. Chem.*, **233**, 350 (1958)
- (3) Sauer, G., and Rapoport, S., *Biochem. Z.*, **331**, 522, 534 (1959)
- (4) Hashimoto, T., Nakao, M., and Yoshikawa, H., *J. Biochem.*, **48**, 158 (1960)
- (5) Towne, J. C., Rodwell, V. W., and Grisolia, S., "Biochem. Preparations" Vol. **6**, 12 (1958)
- (6) Kiessling, W., *Ber. deut. chem. Ges.*, **68B**, 243 (1935)
- (7) Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1941-2)
- (8) Bennett, H. S., and Luft, J. H., *J. Biophys. Biochem. Cytol.*, **6**, 113 (1959)
- (9) Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925)
- (10) Bartlett, G. R., *J. Biochem.*, **234**, 496 (1959)
- (11) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265, (1951)
- (12) Cowgill, R. W., "Methods in Enzymology," Vol. **3**, Academic Press, Inc., New York, 1957, p. 120; *Biochim. et Biophys. Acta.*, **16**, 614 (1955)
- (13) Porath, J., and Flodin, P., *Nature*, **183**, 1657 (1959)
Bjork, W., and Porrrh, J., *Acta Chem. Scand.*, **13**, 1256 (1959)
- (14) Sutherland, E. W., Posternak, T., and Cori, C. F., *J. Biol. Chem.*, **181**, 153 (1949)
- (15) Manyai, S., and Varady, Z., *Biochim. et Biophys. Acta*, **20**, 594 (1956); *Acta Physiol. Acad. Sci. Hung.*, **14**, 153 (1958)

Fate of Radioactive Tin in Rats*

By SHIGEKO MORIMOTO and YOSHIMASA YONEYAMA

(From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine,
University of Tokyo, Tokyo)

(Received for publication, February 28, 1961)

Canned fruit juice often contains a trace amount of tin which originates from the coating of the inner surface of the can. It was desired to obtain information about the absorption and excretion of such small amounts of the element when ingested by mouth. It has been reported that tin is normally present in a slight amount in man (1) and animal (2) bodies, but whether it is only a contaminant or has some physiological function is unknown. The metabolism of tin in organisms (3-6) has been reported, but with discrepancies, and the pharmacological action (7-9) of this metal has been discussed by others. The usual procedures for the determination of tin in organisms (10) are quite complicated, and for the investigation of the metabolism of such minute quantities of tin, a radioactive isotope would be most convenient. In the present work, Sn^{113} was used in the study of the absorption, excretion and distribution of this metal in rats. The administration was made intravenously or per os, as solid diets or through a gastric tube.

EXPERIMENTALS

Animals—Male and female rats, of approximately 100 g. weight, nourished with solid diet (Oriental Yeast Co. MF. Lt. 97), were used as experimental animals.

Radioactivity Measurement of Sn^{113} —The amount of radioactive tin was determined by the gamma ray emitted from $\text{In}^{113\text{m}}$, which is the daughter element in equilibrium, with Sn^{113} , using a well-type scintillation counter. Sn^{113} was supplied as SnCl_2 in dilute HCl. The radiochemical purity of this preparation was 95 per cent as determined by column chromatography Dowex 1 (X4 chloride form)-formate system

(11). The chief contaminants were Sb^{125} and Te^{125} . The radioactivity measurements showed that $\text{In}^{113\text{m}}$ reached equilibrium with Sn^{113} in a minimum of 24 hours after the separation of these two elements with column chromatography. In actual animal experiments, however, no change of radioactivity was observed between the measurements directly after the excision of an organ and those 24 hours later on the same sample, indicating that $\text{In}^{113\text{m}}$ was already in equilibrium with its mother element before the first measurement.

The Sn content of the original Sn^{113} solution was colorimetrically assayed with phenylfluorone**.

Feeding of Sn^{113} —The Sn^{113} solution was adjusted to pH 5 with NaOH and Na-citrate. For injection experiments, isotonicity was maintained by the addition of NaCl. Non-radioactive stannous chloride was added as a carrier in some cases.

Oxidized Sn^{113} solution was prepared as follows: to a SnCl_2^{113} solution was added a slight excess of 3% H_2O_2 to oxidize the Sn^{++} . After heating at 60°C in a water bath to destroy the remaining H_2O_2 , the solution was centrifuged at 3,000 r.p.m. for ten minutes. The supernatant was adjusted to pH 5 with Na citrate.

a) *Per Os Feeding with Solid Diet*—A suitable quantity of Sn^{113} was mixed with water and diluted to 100 ml. To this radioactive solution were added 80 g. of the diet powder described above and 20 g. of starch. The viscous mixture was heated and dried at 60°C for 4 hours and divided into 100 parts. A mean values of radioactivity for each piece was calculated from measurements of five individual ones. Each piece was given to a fasted rat early in the morning during the experimental period of one to three months for complete administration. Water and diet were given *ad libitum*. The animals were killed 7 days after the last isotope administration. Negligible amounts of radioactivity were found in feces.

b) *Single Administration through a Stomach Tube*—Radioactive Sn^{113} solution was given through a vinyl

* The preliminary work has already been reported (1).

** Miura, S., personal communications.

tube connected with an injection syringe directly into the stomach.

c) *Injection*—From 0.5 ml. to 1.0 ml. of isotonic Sn^{113} , divalent or tetravalent, was injected into the femoral vein.

Radioactive Measurement of Tissues and Organs—The animals were killed by bleeding from the carotid artery under anesthesia. Whole or parts of organs were taken into the bottom of a test tube for γ ray measurements. The radioactivity was expressed as c.p.m. per each organ as a whole or per unit weight. The total amounts of bone, blood and muscle were calculated as 17, 5 and 33 per cents respectively, of the total body weight of a rat. The radioactivity of bone per unit weight was a mean value for os mandibularis, femur and skull, while that of muscle was an average of gastrocnemius and back muscles. In experiment of long duration, the correction for the decay of Sn^{113} was taken into consideration.

RESULTS

Absorption and Excretion

a) *Daily Oral Administration in Solid Diets*—The absorption of Sn^{113} after one to three month administration is shown in Table I. The increase of the amount of administered tin or the elongation of experimental period had little effect on tin absorption.

b) *Single Administration of Sn^{113} Solution through a Gastric Tube*—The results are shown in Table II. Most of the tin was excreted in feces, while tiny amount was found in urine. The time course of excretion was shown in Fig. 1. In urine as well as feces, the large part of tin was excreted within 3 days after administration.

c) *Single Injection of (divalent) Sn^{113}* —The

TABLE I

Absorption and Excretion of Daily Oral Administration of Tin in Solid Diets

Animal No.	Administration Period (days)	Administered Amount of Tin Per Day ($\mu\text{g.}$)	Administered Radioactivity per Day (c. p. m.)	Amount Remained in Bodies	
				Total Activity (Excluding Aliment. Canal) (c. p. m.)	Proportion of Admin. Radio-activity %
O 1	28	0.8	3,200	3,040	3
O 2	28	0.8	3,200	360	0.4
O 3	28	3.5	14,300	2,140	0.5
O 4	30	106	58,000	2,970	0.17
O 5	41	106	58,000	3,670	0.16
O 6	61	108	16,600	1,520	0.15
O 7	97	109	31,600	2,700	0.088
O 8	97	109	31,600	1,750	0.057

TABLE II

Absorption and Excretion by Single Administration through a Gastric Tube

Animal No.	Living Period after Administration (days)	Residual Amount (A)		Amount Excreted in Urine (B)		Amount Excreted in Feces (C)		(A+B+C)	
		Activity (c.p.m.)	Proportion to Administered Radioactivity (%)	Act. (c.p.m.)	Proport. (%)	Act.	Proport. (%)	Act. (c.p.m.)	Proport. (%)
S 1	20	2,150	0.9	211,000	89.3	2,550	1.2	216,000	91.5
S 2	20	2,910	1.2	156,000	66.4	2,240	0.9	161,000	68.5
S 3	12	3,650	1.6	173,000	73.4	3,540	1.5	180,000	76.5

Each rat was given the solution containing 235,000 c.p.m. of Sn^{113} and 35 $\mu\text{g.}$ of Sn.

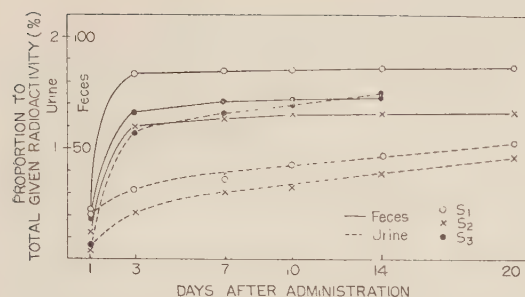


FIG. 1. Time course of cumulative Sn^{113} excretion after administration through a gastric tube.

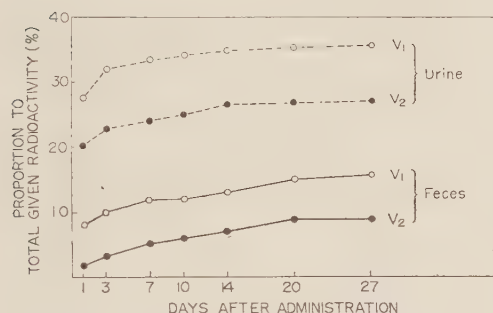


FIG. 2. Time course of cumulative Sn^{113} excretion after (divalent) Sn^{113} injection.

results are given in Table III and Fig. 2. The amount of excretion is maximum in the first day. The residual amount was 30-37 per cent.

d) *Single Injection of Tetravalent Sn^{113}*

The results are shown in Table IV and Fig. 3. The retention of Sn^{113} was larger than that of the former cases.

Distribution among Organs

a) *Daily Oral Administration in Solid Diets*

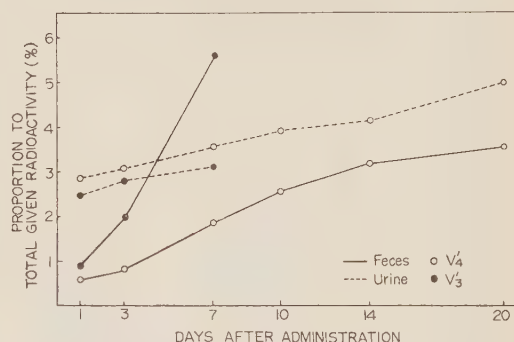


FIG. 3. Time course of cumulative Sn^{113} excretion after Tetravalent Sn^{113} injection.

TABLE III

Retention and Excretion of Intravenous Injection of (Divalent) Sn^{113}

Animal No.	Living Period after Administration (days)	Residual Amount (A)		Amount Excreted in Urine (B)		Amount Excreted in Feces (C)		(A+B+C)	
		Activity (c.p.m.)	Proportion to Administered Rad. (%)	Act. (c.p.m.)	Proport. (%)	Act. (c.p.m.)	Proport. (%)	Act. (c.p.m.)	Proport. (%)
V 1	27	76,100	30.5	41,300	16.5	88,800	35.5	206,000	82.4
V 2	28	91,500	36.6	24,800	10.0	69,100	27.7	185,400	74

Into each rat was injected the solution containing 250,000 c.p.m. of Sn^{113} and 38 μg . of Sn.

TABLE IV

Retention and Excretion of Intravenous Injection of Tetravalent Sn^{113}

Animal No.	Living Period after Administration (days)	Residual Amount (A)		Amount Excreted in Urine (B)		Amount Excreted in Feces (C)		(A+B+C)	
		Activity (c.p.m.)	Proportion to Administered Rad. (%)	Act. (c.p.m.)	Proport. (%)	Act. (c.p.m.)	Proport. (%)	Act. (c.p.m.)	Proport. (%)
V' 3	7	40,300	67.1	2,130	3.6	3,020	5.0	45,400	75.7
V' 4	20	35,400	58.9	3,360	5.6	1,860	3.1	40,600	67.6

Into each rat was injected the solution containing 600,000 c.p.m. of Sn^{113} and 11 μg . of Sn.

TABLE V
Distribution Pattern of Daily Oral Administration

Animal No.	O 2		O 2		O 3		O 4		O 5		O 6		O 7		O 8	
Administered Period (days)	28		28		28		28		41		41		97		97	
Administered Radio-activity per Day (c.p.m.)	3,200		3,200		14,300		14,300		58,000		16,000		31,600		31,600	
Names of Organs	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.
Liver	76	2	36	1	100	2	181	4.6	255	8.5	103	2.7	88	2	104	1.8
Spleen	11	7	15	5	2	0.5	15	6.3	46	34	8	2.3	11	3.4	6	2.2
Kidney	21	5	13	1	9	0.8	43	8.0	45	9.8	42	3.9	42	7.5	32	5.3
													59	9.7	33	5.0
Adrenal	11	18	14	35	8	16	22	55	32	79	5	8.3	2	4.0	0	0
Lung	11	3	0	0	25	3	19	5.8	37	13.4	15	1.6	22	6.8	16	5.5
Heart	8	3	15	3	26	4	13	2.3	38	8.5	14	2.5	24	4.7	14	2.6
Pancreas	16	4	18	3	12	2	16	4.6	32	9.8	10	1.7	15	3.5	9	1.4
Esophagus	17	12	10	7	—	—	26.5	29	32	24	9	7.1	20	15.4	20	9.5
Stomach	55	4	9	0.6	9	0.8	20	1.7	45	4.9	0	0	23	1.5	4	0.4
Intestine	1,210	18	86	2	113	—	192	2.8	4,680	82	100	1.0	149	2.0	122	1.6
Submaxillary gland	18	5	2	0.8	29	9	1.2	0.7	31	12.2	9	3.0	19	12.7	8	5.3
Tongue	15	3	3	0.6	12	2	5.8	2.3	37	14.6	7	2.0	10	2.2	21	4.3
Brain	18	1	16	1	8	0.6	4.6	0.5	38	2.4	10	0.7	37	2.8	14	1.2
Bone	162	2	41	0.5	273	3	930	4.6	1,180	8.5	546	2.2	10	2.0	462	1.8
Muscle	2,260	6	63	0.8	1,290	3	1,400	3.5	1,320	4.9	389	0.8	1,000	2.0	855	1.7
Blood	12	0.2	56	1	39	0.6	81	1.3	108	2.7	—	—	96	1.2	45.6	0.6
Skin	400	2	68	0.4	300	3	240	1.5	475	3.6	364	1.4	775	3.1	128	0.5

TABLE VI
Distribution Pattern of Single Administration through a Stomach Tube

Animal No.	S 1		S 2		S 3	
Living Period after Administration (day)	20		20		14	
Names of Organs	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.
Liver	110	4.5	125	2.3	170	3.4
Spleen	33	18	24	11.2	40	15
Kidney	30	3.5	29	4.9	61	9
Adrenal	26	65	37	92	19	38.6
Lung	48	17	22	3.4	24	1.1
Heart	16	4.5	24	3.4	21	2.3
Pancreas	32	11	16	2.3	31	11
Esophagus	17	18	19	12.3	0	0
Stomach	14	2.3	18	1.1	18	1.1
Intestine	230	16	153	11	210	16
Submaxillary gland	18	5.7	35	6.8	21	5.7
Tongue	15	6.8	24	5.7	34	12.7
Brain	23	1.7	24	1.1	27	2.3
Bone	640	4.5	820	3.4	985	4.5
Muscle	640	2.3	1,030	2.3	1,450	3.4
Blood	68	1.8	21	0.34	68	1.8
Skin	450	2.9	680	3.4	700	3.4

Experimental conditions were described at Table II.

TABLE VII
Distribution Pattern of Intravenous Injection of (Divalent) Sn^{113}

Animal No.	V 1		V 2	
Living Period after Administration (day)	27		28	
Names of Organs	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.
Liver	1,580	44	1,580	36
Spleen	41	41	75	75
Kidney	374	68	613	51
Adrenal	35	70	37	74
Lung	41	12	51	14
Heart	46	13	44	8
Pancreas	54	16	56	13
Esophagus	31	63	34	23
Stomach	29	2.3	50	3.5
Intestine	300	4.6	430	5.8
Submaxillary gland	—	—	42	14
Tongue	35	8	35	7
Brain	30	2.3	29	2.3
Bone	69,000	410	84,400	445
Muscle	3,060	9.3	2,150	5.8
Blood	164	3.7	175	3.5
Skin	1,300	16	1,660	10.2

Experimental conditions were described at Table III.

TABLE VIII

Distribution Pattern of Intravenous Injection of Tetravalent Sn¹¹³

Animal No.	V' 1		V' 2		V' 3		V' 4	
Living Period after Administration (day)	0.25		0.5		7		20	
Names of Organs	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.	c.p.m. whole organ	c.p.m. 100 mg.
Liver	44,200	1,110	33,100	570	32,800	664	31,000	646
Spleen	514	514	206	425	157	433	188	791
Kidney	110	20	72	11	61	11	71	12
	109	20	70	11	60	10	64	10
Adrenal	61	152	52	104	38	126	28	70
Lung	166	41	118	42	601	111	45	12
Heart	120	25	35	7	113	25	22	6
Pancreas	49	18	66	17	32	13	27	9
Esophagus	107	82	30	25	16	13	10	13
Stomach	107	8	175	10	24	2	221	18
Intestine	534	8	441	5	820	12	147	2
Submaxillary Gland	9	9	15	4	35	32	17	10
Tongue	16	4	—	—	7	2	12	3
Brain	26	2	2	0.1	18	2	14	1
Bone	2,370	11	2,720	11	3,320	17	1,380	7
Muscle	1,270	3	966	2	1,530	4	1,170	3
Blood	252	4	146	2	58	1	58	1
Skin	1,470	7	440	2	320	2	288	2

Experimental conditions were described at Table IV.

TABLE IX

Site of Excretion of Sn¹¹³ after Intravenous Injection

Animal No.	V'' 1		V'' 2		V'' 3		V'' 4		V'' 5		V'' 6							
Injected Radioactivity							170,000 c.p.m.						50,000 c.p.m.					
Living Period after Injection (hours)	1		5		10		21		168		480							
	c.p.m. whole organ	c.p.m. 100mg.	c.p.m. whole organ	c.p.m. 100mg.	c.p.m. whole organ	c.p.m. 100mg.	c.p.m. whole organ	c.p.m. 100mg.	c.p.m. whole organ	c.p.m. 100mg.	c.p.m. whole organ	c.p.m. 100mg.						
Esophagus	8	13	32	46	9	13	12	17	18	5	1	0.2						
Stomach	95	11	478	39	159	12	108	9	19	3	33	4						
Intestine 1	19	4	65	26	166	33	46	16	42	5	15	7						
2	45	4	139	23	63	10	67	9	33	4	16	2						
3	163	13	490	38	79	13	82	12	—	—	—	—						
4	137	13	150	23	56	10	96	16	25	4	2	4						
5	113	11	220	38	324	26	163	15	—	—	—	—						
6	108	31	417	73	120	16	223	33	115	22	8	1						
(Cecum) 7	20	4	1,660	85	404	24	814	48	135	21	19	2						
8	25	3	217	34	46	84	31	84	25	5	21	3						
9	19	4	27	12	10	4	194	43	21	12	—	—						

As shown in Table V, no characteristic distribution pattern was obtained. With longer experiments this trend did not change. The most radioactive organ per unit weight was the adrenals.

b) Single Administration of Sn^{113} Solution through a Gastric Tube—The results were similar to those of case (a) as shown in Table VI.

c) Single Injection of (divalent) Sn^{113} —Bone was the tissue richest in Sn^{113} as shown in Table VII. A small amount of tin was found in the intestine, a fact which might indicate excretion of this metal by this route.

d) Single Injection of Tetravalent Sn^{113} —Most of the injected tin was found in the reticulo-endothelial organs. (Table VIII) No change of distribution pattern was observed with the length of the experimental period.

Site of Excretion in Intestine after Single Injection of (divalent) Sn^{113}

The excretion of Sn^{113} in different parts of alimental canal after a single injection of Sn^{113} is shown in Table IX. The intestine was divided into 9 nearly equal length. The lower parts of the alimental canal showed relatively high activity in the early experimental period. No radioactivity was found in blood six hours after injection.

DISCUSSION

The large part of tin administered per os was excreted and unabsorbed. That heavy metals are absorbed with difficulty (12) as compared with alkaline earths is a well known fact. Tin might be a typical example of these heavy metals. We could assume that most of the solubilized tin in canned juice also is unabsorbed and excreted in the feces. But our experiments clearly showed that tin was, though in tiny amounts, absorbed and then excreted. In the injection experiments, more Sn^{113} was found in urine than in feces (except one case) which is similar to the case of Mn excretion (12). The relatively high Sn^{113} content of the lower intestine in the early period is to be studied further to determine whether it results from excretion or retention.

No characteristic distribution of tin among organs was found in oral feeding, while injected reduced Sn^{113} was mainly found in bone and the oxidized metal in the reticulo-endothelial system. In every case the adrenals accumulated high radioactivity, the significance of which is not easy to explain. Divalent tin might be deposited in bone in place of calcium ion whereas colloidal tetravalent tin particles be captured by reticulo-endothelial organs, as demonstrated in the case of gold, iron and other colloidal solutions. That the retention of tin was larger in the oxidized Sn^{113} injection was also in accordance with the accumulation of this metal in the reticulo-endothelial system. Different administration methods gave different distribution patterns. In order to explain this fact the possibility of redistribution of tin via blood vessels was considered, for the presence of tin in human blood has been reported. (1a), and in the case of poisoning with lead, an element in the same family as tin, lead is believed first to accumulate in the visceral organs and then to move to bone (13). The time course study of the distribution pattern was to determine if such a redistribution might take place, but in no case, oral or injection, was a change in distribution pattern observed. Tin seems to be a less mobile element. If this element is captured by tissues or organs, it is difficult to remove. But, tin is really excreted and has been reported to be in blood. These discrepancies are to be studied further.

SUMMARY

1. The absorption, excretion and distribution of Sn^{113} were studied in rats after per os feeding and injection. Almost all of the administered tin was not absorbed. A small part was absorbed and then excreted.

2. The injected tin was more excreted into urine in larger amount than into feces. The injected divalent Sn^{113} was accumulated in bone, whereas the tetravalent metal in the reticulo-endothels. High radio-activity was found in the lower intestine shortly after the injection of Sn^{113} .

Thanks are due to Prof. H. Yoshikawa for his helpful discussions.

REFERENCES

- (1) Morimoto, S., *J. Japan. Biochem. Soc.*, **31**, 212 (1959)
- (1a) Kehoe R. A., Cholak J., and Story R. V., *J. Am. Med. Assoc.*, **104**, 90 (1935)
- (2) Bertrand, G., and Civrea, V., *Compt. Rend. Acad. Sci.*, **192**, 780 (1931)
- (3) Kent, N. L., and Mc Cane, R. A., *Biochem. J.*, **35**, 877 (1941)
- (4) Salant, W., Rieger, J. B., and Tseuthardt, E. L. P., *J. Biol. Chem.*, **17**, 265 (1914)
- (5) Salant, W., Rieger, J. B., and Tseuthardt, E. L. P., *J. Biol. Chem.*, **34**, 463 (1918)
- (6) Misk, E., *Compt. Rend. Acad. Sci.*, **176**, 138 (1923)
- (7) White, J. P., *Arch. Exptl. Path.*, **13**, 53 (1881)
- (8) Lehmann, *Arch. Hyg.*, **45**, 88 (1902)
- (9) Schwarze, E. W., and Clarke, W. C., *J. Pharmacol.*, **31**, 224 (1927)
- (10) Sandell, E. B., "Colorimetric Determination of Traces of Metals", Interscience Publishers, Inc., New York, p. 852 (1959)
- (11) Hashimoto, H., Morimoto, S., and Yoneyama, Y., unpublished.
- (12) Greenberg, D. E., Copp, D. H., and Cuthbertson, E. M., *J. Biol. Chem.*, **147**, 749 (1943)
- (13) Cantarow, A., and Trumper, M., "Lead Poisoning", Williams & Wilkins Co., Baltimore (1944) cited by Goodman, L. S., and Gilman, A., "The Pharmacological Basis of Therapeutics", Macmillan Co. New York p. 1002 (1958)

Zur Beeinflussung des Ubichinongehaltes der Leber wachsender Ratten durch verschiedene Diätformen

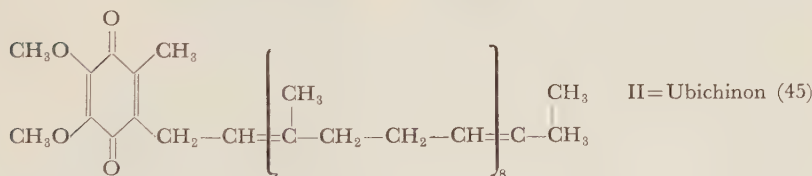
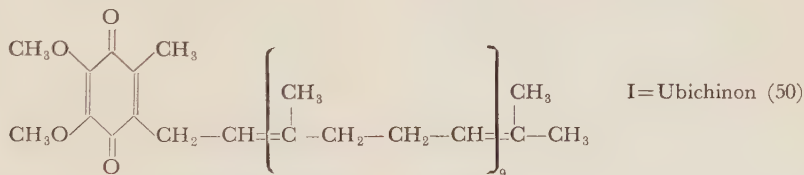
Von O. WISS und G. BRUBACHER

(aus der Abteilung für Vitamin- und Ernährungsforschung der F. Hoffmann-La Roche & Co.
A. G. Basel, Schweiz)

(Der Schriftleitung zugegangen am 22 April 1961)

Kürzlich haben U. Gloor, O. Schindler und O. Wiss (1) gezeigt, dass der Rattenorganismus imstande ist, die ganze Seitenkette von Ubichinon(50) resp. Ubichinon(45) (Formeln I und II) zu synthetisieren. In einer weiteren Arbeit konnte ferner nachgewiesen werden, dass die Methylradikale der beiden kernständigen Aethergruppen ebenfalls im Tierkörper eingeführt werden, wobei Methionin als Methylendonator dient (2). Der Ursprung des benzochinoiden Kernes hingegen ist vorläufig noch ungewiss.

lösliche Vorstufe nachweisen zu können, verschiedene Diätformen an wachsende Ratten verfüttert und nach bestimmten Zeitabständen die Leber der Tiere auf ihren Ubichinongehalt analysiert. Dabei gingen wir von der Arbeitshypothese aus, dass bei einer Diät, welche die unbekannte Vorstufe in erhöhtem Masse enthalten würde, sich dies daran zu erkennen geben sollte, dass der Ubichinongehalt der Leber in entsprechendem Ausmass gesteigert würde. Im folgenden sei über die durchgeführten Versuche berichtet.



Die Tatsache, dass strukturverwandte Verbindungen, wie Vitamin E und K, dem Organismus zugeführt werden müssen, ohne dass dieser in der Lage wäre, dieselben zu synthetisieren, lässt vermuten, dass auch im Falle von Ubichinon eine Verbindung zugeführt werden muss, in welcher der benzochinoiden Kern bereits vorgebildet ist. Eine solche Verbindung muss nicht unbedingt lipidlösliche Eigenschaften aufweisen, da ja Seitenkette und Methylgruppen erst im tierischen Organismus angefügt werden. Wir haben daher, um eine solche eventuell wasser-

EXPERIMENTELLES UND RESULTAT

Versuchstiere—Für alle Versuche wurden Wistar-Ratten aus eigener Zucht verwendet. Die Tiere wurden mit 3 Wochen abgesetzt, sie kamen anschliessend in Einzelkäfige und erhielten die angegebenen Spezialdiäten.

Diäten—Die von uns verwendeten Diäten beruhen auf systematischer Abwandlung der von M. O. Schultze (3) angegebenen Formeln. Die Diäten wurden mindestens alle 10 Tage frisch gemischt, ihre Zusammensetzung geht aus Tabelle I hervor.

TABELLE I
Rattendiäten (Angaben in Gramm)

Bestandteil	S 1116	S 1117	S 1118	S 1119	S 1121	S 1122	S 1123
Saccharose	74.546						
Glucose		74.546			74.546	74.546	74.546
Fructose			74.546				
Reisstärke				74.546			
Casein (Vitaminfrei)	17.84	17.84	17.84	17.84	18.140		
Lactalbumin						8.0	
Gelatine						10.0	
Soya- α -Protein							18.140
DL-Methionin	0.3	0.3	0.3	0.3			
L-Histidin-mono-HCl						0.140	
Salzmischung I	4	4	4	4	4	4	4
Zucker-Vitamin-Mischung 12	2	2	2	2	2	2	2
Rovimix A+B ₂ +D ₃	0.024	0.024	0.024	0.024	0.024	0.024	0.024
Rovimix E	0.040	0.040	0.040	0.040	0.040	0.040	0.040
Methylinoleat-Harnstoff-Komplex	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Total :	100 g	100 g	100 g	100 g	100 g	100 g	100 g

Für die Herstellung der Diäten verwendeten wir möglichst reine Handelsprodukte. Als Lactalbumin diente das Lactalbumin der Fa. Nutritional Biochemicals Corporation (Cleveland/Ohio), das im Soxhlet mit Aceton erschöpfend extrahiert wurde. Gelatine wurde ebenfalls im Soxhlet erschöpfend extrahiert. Bei Soya- α -Protein handelt es sich um ein Produkt der Central Soya-Company Inc. (1825 N Laramie Avenue, Chicago 39/111.).

Die Salzmischung enthält :

Natriumchlorid	5.00 %
Calciumlacetat	35.00 %
Tricalciumphosphat	15.00 %
Eisen(III)citrat	3.21 %
Kaliumjodid	0.09 %
Kupfersulfat	0.03 %
Magnesiumsulfat	5.50 %
Kaliumphosphat	26.527%
Natriumphosphat	9.60 %
Zinkcarbonat	0.02 %
Mangansulfat	0.02 %
Natriumfluorid	0.003%

Die Zucker-Vitamin-Mischung enthält :

Thiaminhydrochlorid	250 mg
Pyridoxin	250 mg

Calciumpantothenat	2,500 mg
Nikotinsäureamid	1,000 mg
Inosit	20,000 mg
Folsäure	10 mg
Vitamin B ₁₂ -Konzentrat	2,000 mg
Menadion	250 mg

Diese Mengen werden in 973.74 g Rohrzucker eingemischt. Das Vitamin B₁₂-Konzentrat bestand aus einer Mischung von 1 mg. Vitamin B₁₂ und 1 g D-Mannit.

Rovimix A, B, D₃, resp. Rovimix E sind stabile Vitamintrockenformen. Rovimix A, B₂, D₃ enthält 40,000 IE. Vitamin A, 40 mg Vitamin B₂ und 10,000 IE. Vitamin D₃ pro Gramm Trockenpulver.

Rovimix E enthält 250 mg DL- α -Toko-pherolacetat pro Gramm Trockenpulver. Der Methylinoleat-Harnstoffkomplex wurde durch Zusammengeben von 125 g Linolsäuremethylester und 500 g Harnstoff in wenig Methanol hergestellt.

Zu je 1 kg Diät wurden unmittelbar vor Gebrauch 1 g Cholinchlorid eingemischt. Die Diätmischungen wurden in verschlossenen Büchsen bei ca. 4°C aufbewahrt.

Analytik—Zur Bestimmung des Ubichinon-

gehaltes der Leber haben wir eine Farbreaktion verwendet, die Ubichinon mit Essigsäureanhydrid in Schwefelsäure-Eisessig eingeht. Diese Farbreaktion ist sehr spezifisch, indem unter den gewählten Versuchsbedingungen 500 μg folgender Verbindungen folgende Extinktionen ergaben:

DL- α -Tokophrol	nicht messbar
DL- α -Tokopherolacetat	do.
Vitamin K ₁	do.
Pflanzliches Chinon (4)	do.
Solanesol	0.001
Cholesterin	0.004

500 μg Ubichinon (50) dagegen zeigen unter den Versuchsbedingungen eine Extinktion von 0.117, 500 μg Ubichinon (45) eine solche von 0.135, 500 μg Ubichinon (30) eine solche von 0.165 und eine synthetische Verbindung, die anstelle der Ubichinon-Seitenkette die Phetyl-Seitenkette aufweist (5), eine solche von 0.258.

Die mit den Verbindungen der Ubichinonreihe entstehende Blaufärbung ist äusserst flüchtig und verschwindet bereits nach zwei Minuten. Die nach 30 Sek. abgelesene Extinktion folgt im Bereich von 200–1000 μg dem Lambert-Beer'schen Gesetz. Im Gegensatz zum Verhalten von Ubichinon gibt Vitamin A unter den Reaktionsbedingungen eine Blaufärbung, die auch nach zwei Minuten bestehen bleibt. Die Anwesenheit von Vitamin A ergibt sich damit leicht zu erkennen. Bei Anwesenheit von Vitamin A muss eine chromatographische Reinigung eingeschaltet oder auf die Verwendung der Farbreaktion verzichtet werden. Als praktische Ausführung haben wir folgendes Vorgehen gewählt:

Bestimmung von Ubichinon in der Leber

1.) *Verseifung der Leber*—Ein aliquoter Teil einer Leber, der mindestens 200 μg Ubichinon enthalten soll, wird mit dem gleichen Volumen methanolischer KOH (a) und einigen Kristallen Pyrogallol (b) 15 Minuten auf dem kochenden Wasserbad erhitzt, man fügt 20 ml Methanol (c) und wenige ml Wasser hinzu, bringt in einen Scheidetrichter und spült mit ca. 30–40 ml Wasser nach. Man schüttelt mit je 100 ml Aether (d) zwei mal aus, vereinigt die ätherischen Phasen,

wäscht mit Wasser bis zur neutralen Reaktion, trocknet mit Na_2SO_4 (e) und bringt nach Filtration im Vakuum zur Trockne.

2.) *Chromatographische Trennung von Vitamin A*.—Das Unverseifbare der Leberlipide wird in 5 ml Cyclohexan (f) aufgenommen und an einer Aluminiumoxydsäule chromatographiert, die dreissigmal das Gewicht des Unverseifbaren an Al_2O_3 (g) enthält. Man bringt die Substanz auf und spült mit 20 ml Cyclohexan nach, dann wird mit 20 ml Cyclohexan-Aether 2% (h) eluiert, das Eluat am Vakuum eingedampft und nochmals an einer Säule, enthaltend 1 g Al_2O_3 (g), chromatographiert. Die mit Cyclohexan-Aether 2% (h) eluierte Fraktion zeigt im Gebiet des Ubichinonmaximums beinahe keine Fremddabsorption; der Ubichinongehalt kann darin entweder spektrophotometrisch oder kolorimetrisch bestimmt werden.

3.) *Bestimmung*: a) *Kolorimetrische Bestimmung von Ubichinon*.—Der Rückstand, der mindestens 200 μg Ubichinon enthalten soll, wird mit 2 ml einer Mischung von konzentrierter Schwefelsäure und Eisessig (i) gelöst, man fügt 4 ml Essigsäureanhydrid (k) hinzu und liest die Extinktion der erhaltenen Lösung nach genau 30 Sekunden bei 570 $m\mu$ ab, wobei eine Lösung von 2 ml konzentrierter Schwefelsäure-Eisessig (i) und 4 ml. Essigsäureanhydrid (k) als Blindwert dient.

b) *Spektrophotometrische Bestimmung*.—Der ubichinonhaltige Rückstand wird in hochsiedenden Petroläther (l) aufgenommen und das Absorptionsspektrum der Lösung zwischen 220 $m\mu$ und 360 $m\mu$ bestimmt. Der Ubichinongehalt errechnet sich dann unter Zugrundelegung eines $E_{1\text{cm}}^{1\%}$ -Wertes von 170 für eine Wellenlänge von 272 $m\mu$.

Die spektrophotometrische Methode gibt in der Regel niedrigere Analysenwerte als die kolorimetrische Methode. Unter normalen Umständen liegen die beiden Analysenwerte jedoch höchstens 30% voneinander. Sollte der spektrophotometrische Wert mehr als 30% höher liegen, so muss dies auf Fremddabsorption zurückgeführt und der spektrophotometrische Wert verworfen werden. Andererseits kann das Spektrum durch Auftreten eines

Vitamin A anzeigen. In diesem Fall muss der kolorimetrisch bestimmte Wert verworfen werden.

4.) Reagentien

- a) Methanolische KOH: 25 g KOH werden in absolutem Methanol aufgelöst und auf 100 ml aufgefüllt;
- b) Pyrogallol: Handelsware
- c) Methanol: absolut
- d) Aether: absolut
- e) Na_2SO_4 : Handelsware
- f) Cyclohexan: reinst. für Chromatographie.
Käufliches Cyclohexan kann eine bei ca. 270 m μ absorbierende Verunreinigung enthalten und sollte aus diesem Grunde vor Gebrauch destilliert werden.
- g) Al_2O_3 : 100 g Aluminiumoxyd "Giulini", Aktivitätsstufe I, werden mit 7 ml Wasser desaktiviert.
- h) Eluiergemisch: Cyclohexan-Aether 2%, 98 ml Cyclohexan werden mit 2 ml Aether gemischt.
- i) Schwefelsäure-Eisessig: 4.9 g Schwefelsäure konzentriert, pro anal. werden mit Eisessig pro anal. auf 100 ml aufgefüllt.
- k) Essigsäureanhydrid: mindestens 98%-ig.
- l) Petroläther: optisch rein, hochsiedend.

Maximums bei ca. 325 m μ Anwesenheit von

Weder bei der kolorimetrischen, noch bei der spektrophotometrischen Bestimmung wurde zwischen Ubichinon (50) und Ubichinon (45) unterschieden. Der Einfachheit halber wurden die Resultate als Ubichinon (50) berechnet. Die Rattenleber enthält in der Regel neben Ubichinon (50) (ca. 20-30%) in grösseren Mengen Ubichinon (45) (ca. 70-80%) und Spuren der Ubichinone (35) und (40) (6). Nun zeigt Ubichinon (50) einen $E_{1\text{cm}}^{1\%}$ -Wert bei 272 m μ von 168-170, während bei Ubichinon (45) derselbe 186-188 beträgt; das Verhältnis ist somit 0.9. Für die Farbreaktion beträgt das Verhältnis der $E_{1\text{cm}}^{1\%}$ -Werte 0,87. Es sind also innerhalb der Analysengenauigkeit für die spektrophotometrische Methode dieselben Werte zu erwarten wie für die kolorimetrische, unabhängig davon, in welchem Verhältnis die beiden Ubichinone vorliegen.

In dreizehn aufeinander folgenden Bestimmungen von dreizehn verschiedenen Leberproben betrug der Unterschied zwischen

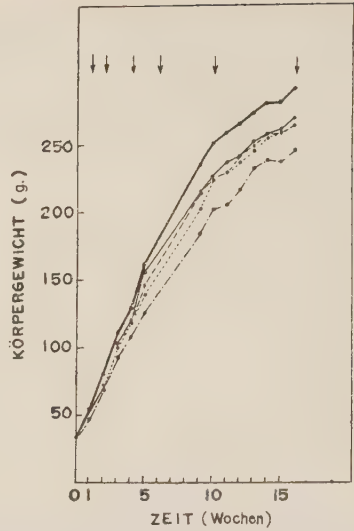


FIG. 1. Gewichtsentwicklung von männlichen Ratten nach Verabfolgung folgender Diäten: An den mit ↓ bezeichneten Stellen wurden jeweils 3 Tiere getötet.
— Diät S 1116 Saccharose, --- Diät S 1117 Glucose, — · — Diät S 1118 Fructose, Diät S 1119 Stärke, —●— Diät S 1116+60 mg Ubichinon(50)/kg.

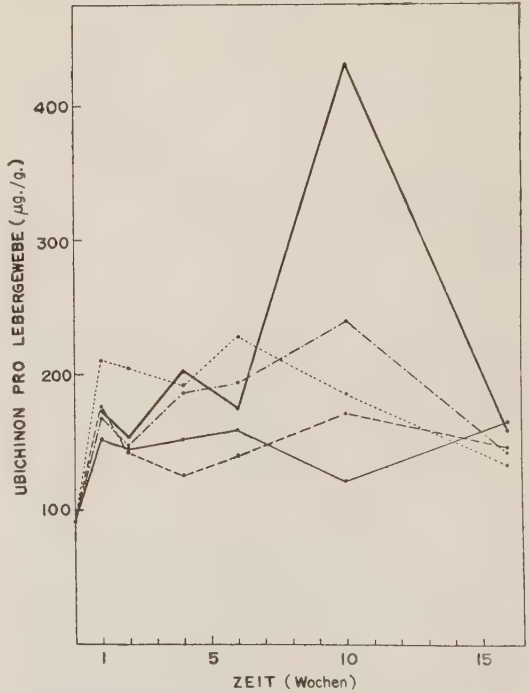


FIG. 2. Ubichinongehalt von Lebern männlicher Ratten nach Verabfolgung folgender Diäten: — Diät S 1116 Saccharose, --- Diät S 1117 Glucose, — · — Diät S 1118 Fructose, Diät S 1119 Stärke, —●— Diät S 1116+60 mg Ubichinon (50)/kg.

kolorimetrischer und spektrophotometrischer Bestimmung: +1.5%, +1.9%, +0.06%, +22%, +15%, +8.4%, +9.1%, +2.0%, +5.7%, 8.4%, +0.6%, +0.09%, +11.9%. Für unsere praktischen Zwecke wurde, soweit die Werte nicht mehr als 30% auseinander liegen, stets der Mittelwert zwischen den beiden Bestimmungsarten gewählt.

Durchführung der Versuche und Ergebnisse

In einer ersten Versuchsserie wurden je zwanzig männliche Ratten im Gewicht von 30–40 g auf unsere Versuchsdiäten S 1116, S 1117, S 1118 und S 1119 gesetzt, als fünfte Gruppe wurde schliesslich eine Gruppe von Tieren gebildet, die zusätzlich mit ihrer Diät S 1116 noch 60 mg Ubichinon(50)/kg Futter erhielt. Im Abstand von 1, 2, 4, 6, 10 und 16 Wochen wurden je drei Tiere getötet. Die Lebern der Tiere wurden herauspräpariert und der Ubichinongehalt bestimmt. In Fig. 1 ist die Gewichtsentwicklung der Tiere, in Fig. 2 sind die Mittelwerte für die Ubichinongehalte in $\mu\text{g/g}$ Leber-Frischgewebe, und in Fig. 3 die Mittelwerte für die totalen

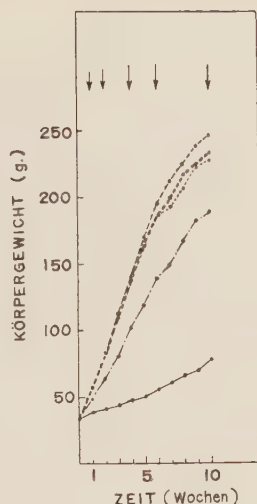


Fig. 4. Gewichtsentwicklung von männlichen Ratten nach Verabfolgung folgender Diäten: An der mit ↓ bezeichneten Stellen wurden jeweils 3 3 Tiere getötet.

--- Diät S 1117 Glucose-Casein-Methionin, --- Diät S 1121 Glucose-Casein, --- Diät S 1122 Glucose-Lactalbumin, — Diät S 1123 Glucose-Soyaprotein, --- Diät S 1117+60 mg Ubichinon (50)/kg.

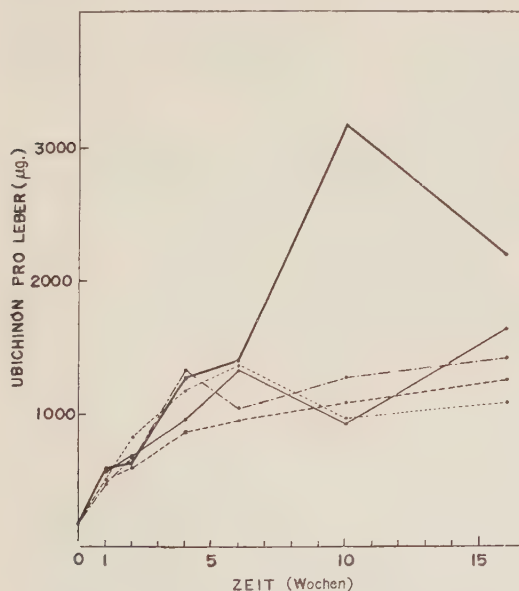


Fig. 3. Ubichinongehalt von Lebern männlicher Ratten nach Verabfolgung folgender Diäten: — Diät S 1116 Saccharose, --- Diät S 1117 Glucose, --- Diät S 1118 Fructose, Diät S 1119 Starke, — Diät S 1116+60 mg Ubichinon (50)/kg.

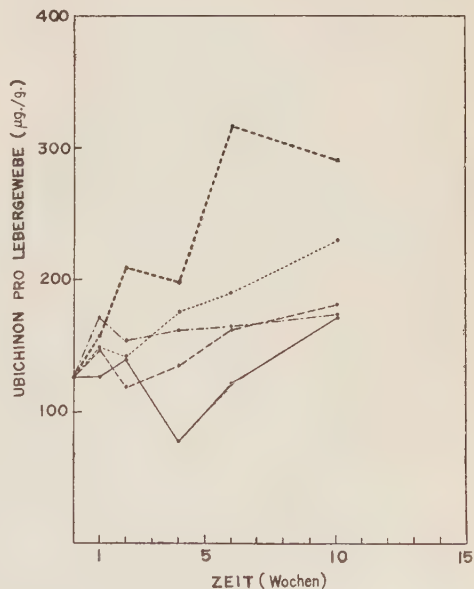


Fig. 5. Ubichinongehalt von Lebern männlicher Ratten nach Verabfolgung folgender Diäten: --- Diät S 1117 Glucose-Casein-Methionin, Diät S 1121 Glucose-Casein, --- Diät S 1122 Glucose-Lactalbumin, — Diät S 1123 Glucose-Soyaprotein, --- Diät S 1117+Ubichinon (50) 60 mg/kg.

Ubichinonmengen dargestellt.

In einer zweiten Versuchsserie wurden wiederum je 20 Tiere im Gewicht von 30-40 g auf unsere Versuchsdiäten S 1117, S 1121, S 1122 und S 1123 gesetzt und eine fünfte Gruppe erhielt wiederum Diät S 1117, der noch 60 mg/kg Ubichinon(50) zugesetzt worden war. Die Tiere wurden in derselben Reihenfolge wie in unserem ersten Versuch getötet und die Lebern auf ihren Ubichinongehalt untersucht. Die Ergebnisse dieser zweiten Versuchsserie sind aus den Figuren 4-6 ersichtlich.

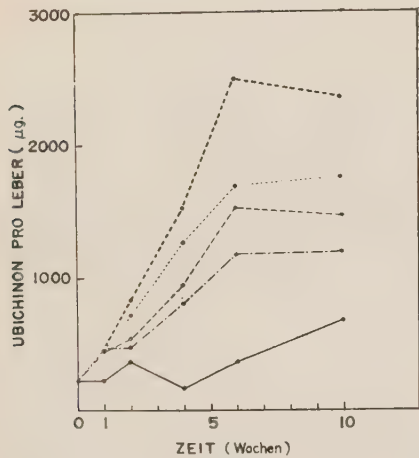


FIG. 6. Ubichinongehalt von Lebern männlicher Ratten nach Verabfolgung folgender Diäten: --- Diät S 1117 Glucose-Casein-Methionin, ... Diät S 1121 Glucose-Casein, -.-.- Diät S 1122 Glucose-Lactalbumin, — Diät S 1123 Glucose-Soyaprotein, --- Diät S 1117+Ubichinon (50) 60 mg/kg.

DISKUSSION

Aus den Figuren 2 und 5 geht hervor,

dass der Ubichinongehalt der Leber innerhalb gewisser Grenzen, ausgedrückt in $\mu\text{g/g}$, bei den Tieren, die kein Ubichinon zusätzlich mit der Nahrung erhielten, konstant ist. Für die Diäten S 1116 (Saccharose-Casein-Methionin), S 1117 (Glucose-Casein-Methionin) und S 1122 (Glucose-Lactalbumin-Gelatine) schwanken die gemessenen Werte zwischen 120 und 180 $\mu\text{g/g}$. Diese Zahlen stellen den Mittelwert von drei Leberwerten dar, die Einzelwerte sind selbstverständlich grösseren Schwankungen unterworfen, doch kamen Einzelwerte, die 100 $\mu\text{g/g}$ unterschritten, resp. 200 $\mu\text{g/g}$ überschritten, nur selten vor.

Für die Diäten S 1118 (Fructose-Casein-Methionin), S 1119 (Stärke-Casein-Methionin) und S 1121 (Glucose-Casein) wurden Werte zwischen 130 und 240 $\mu\text{g/g}$ gefunden; es liegt damit deutlich eine Tendenz für höhere Ubichinongehalte bei diesen Diäten vor, eine Tendenz, die in gewissen Fällen auch statistisch gesichert werden konnte. So gehen die Einzelwerte für die vierte Versuchswoche der ersten Versuchsserie aus Tabelle II hervor.

Eine Streuungszerlegung mit anschliessendem F- resp. t-Test zeigt mindestens 95%ige Wahrscheinlichkeit für eine Signifikanz der Unterschiede zwischen S 1116 resp. S 1117 einerseits und S 1118, S 1119 und S 1116+Ubichinon anderseits an.

Die entsprechenden Einzelwerte für die vierte Versuchswoche der zweiten Versuchsserie gehen aus Tabelle III hervor:

Eine Streuungszerlegung zeigte in diesem Fall keine signifikanten Unterschiede an. Die Versuchsergebnisse in dieser zweiten Versuchsserie streuten erheblich stärker als dieje-

TABELLE II

Einzelwerte für die Ubichinongehalte der Leber der Tiere der ersten Versuchsserie, gemessen nach 4 Wochen Versuchsdauer

Diät:	S 1116 (Saccharose)	S 1117 (Glucose)	S 1118 (Fructose)	S 1119 (Stärke)	S 1116+ Ubichinon
Ubichinongehalt in $\mu\text{g/g}$	142	130	180	181	179
	169	124	180	209	228
	146	120	202	183	198
Mittelwert:	152.3	124.7	197.3	191	201.7

TABELLE III

Einzelwerte für die Ubichinongehalte der Leber der Tiere der zweiten Versuchsserie,
gemessen nach 4 Wochen Versuchsdauer

Diät :	S 1117 (Casein- Methionin)	S 1121 (Casein)	S 1122 (Lactalbumin)	S 1123 (Soyaprotein)	S 1117+ Ubichinon
Ubichinongehalt in $\mu\text{g/g}$	164	251	193	77	235
	84	142	212	80	161
	160	135	172		201
Mittelwert :	136	176	162	78	199

nigen der ersten Versuchsserie, ohne dass wir einen Grund dafür gefunden hätten. Immerhin spricht die Tatsache, dass auch in der 1., 2., 6. und 10. Versuchswoche die Resultate der Diät S 1121 höher lagen als diejenigen der Diät S 1117, sehr für das Vorliegen eines wesentlichen Unterschiedes.

Wie aus den Figuren 3 und 6 hervorgeht, ist die Erhöhung des Leberubichinongehaltes nicht nur relativ, sondern auch absolut vorhanden und nicht etwa durch ein kleineres Lebergewicht zu erklären.

Interessanterweise lagen nach 16 Versuchswochen alle Mittelwerte im Bereich von $132 \mu\text{g/g}$ – $164 \mu\text{g/g}$, wobei vor allem der starke Abfall im Leberubichinongehalt der Tiere interessiert, die mit ihrer Diät Ubichinon (50) zugefüttert erhalten hatten. Es scheint damit, dass der Rattenorganismus über einen Mechanismus verfügt, der ihm gestattet, den Ubichinongehalt innerhalb gewisser Grenzen konstant zu halten.

Der raschere Anstieg in den Ubichinonwerten nach Fructose resp. Stärkezufuhr im Vergleich mit einer Saccharose- resp. Glucosediät könnte auf verschiedene Gründe zurückgeführt werden. Möglicherweise enthält Stärke oder Fructose im Sinne unserer Arbeitshypothese als Verunreinigung eine Vorstufe, die durch Biosynthese im Rattendarm oder im Rattenorganismus in Ubichinon übergeführt werden kann. Es ist jedoch auch denkbar, dass der erhöhte Leberubichinongehalt durch Veränderung der Darmflora oder des Gesamtstoffwechsels bewirkt wird. Schliesslich sei in diesem Zusammenhang an eine Untersuchung von Portman *et al.* (7)

erinnert, wonach der Serumcholesterinspiegel nach Verfütterung einer cholesterinhaltigen Saccharosediat höher ist als nach Verfütterung einer entsprechenden Stärkediät. Die Autoren bringen den Effekt in Zusammenhang mit der erhöhten Ausscheidung von Gallensäuren nach Verfütterung von Maisstärke.

Der erhöhte Ubichinongehalt bei der Diät S 1121 im Vergleich zur Diät S 1117 in der zweiten Versuchsserie kann nicht auf das Vorliegen einer Vorstufe in Diät S 1121 zurückgeführt werden, da sich Diät S 1121 lediglich durch einen kleineren Methioningehalt von Diät S 1117 unterscheidet.

Der Ubichinongehalt von Rattenlebern wurde schon von verschiedenen Autoren untersucht, wobei folgende Befunde gemacht werden konnten:

Vitamin A-Mangel führt zu Erhöhung des Ubichinongehaltes der Leber. Dieser Umstand war bekanntlich bei der Entdeckung der Ubichinone durch R. A. Morton und seine Schule massgeblich beteiligt. Die Verhältnisse wurden kürzlich von T. Moore und I. M. Sharman (8) diskutiert. J. Green *et al.* (9) fanden, dass weder Mangel noch Angebot an Thiamin oder Riboflavin den Ubichinongehalt der Rattenleber zu beeinflussen vermag. Pantothen säuremangel hingegen führte zu Erhöhung des Ubichinongehaltes, im Gegensatz zu Befunden von Aiyar *et al.* (10). Die Verhältnisse bedürfen daher noch weiterer Abklärung.

Vitamin E-Mangel zog ein Absinken des Ubichinongehaltes in verschiedenen Geweben nach sich, während Vitamin E-Zufuhr denselben erhöhte. Auch dieser Befund steht im

Gegensatz zu solchen anderer Autoren (11, 12). Die Verhältnisse müssen daher ebenfalls weiter untersucht werden. Natriumselenitzufuhr (9) und Thyroxinzufuhr (13) führen ebenfalls zu einer Erhöhung des Ubichinongehaltes der Leber, während Vitamin K-Mangel keinen Einfluss auf den Ubichinongehalt von Rattenlebern zu haben scheint (14).

Es wäre bei dem mit Diät S 1121 beobachteten Effekt am ehesten an eine ähnliche Wirkung zu denken, wie sie bei Vitamin A-Mangel oder bei Thyroxinzufuhr auftritt. Merkwürdigerweise konnten wir eine solche Beobachtung jedoch bei Diät S 1123 nicht machen. Diät S 1123 ist eine ausgesprochene Methionin-Mangeldiät (siehe z. B. Olson und Dinning, 15). Das Wachstum der damit gefütterten Tiere ist äusserst schwach, jedoch bewegen sich die gefundenen Leberubichinonwerte gleichwohl zwischen 80 und 175 $\mu\text{g/g}$, also Werten, wie sie auch bei einer Volldiät angetroffen werden. Wir glauben daher, den Einfluss von Methionin auf den Ubichinongehalt der Lebern noch weiter untersuchen zu müssen, bevor wir den gemachten Befund beurteilen können.

Die in unseren Versuchen verwendeten Diäten bestanden zur Hauptsache aus einer Kohlehydrat- und einer Proteinquelle, wobei wir nach Möglichkeit hochgereinigte definierte Substanzen verwendeten. Es ist unwahrscheinlich, dass in all diesen Substraten eine eventuelle biochemische Vorstufe in gleicher Konzentration vorhanden wäre. Unsere Arbeitshypothese, dass sich eine erhöhte Konzentration einer solchen Vorstufe in einer erhöhten Speicherung von Ubichinon in der Leber widerspiegeln würde, ergab lediglich für die Diäten, welche Fructose oder Stärke enthielten, Anhaltspunkte für das Vorliegen einer derartigen Vorstufe. Andererseits zeigte es sich aber, dass der Organismus imstande ist, den Leberubichinongehalt innerhalb gewisser Grenzen unabhängig von der jeweiligen Zufuhr konstant zu halten. Eine solche Tendenz muss aber wohl so gedeutet werden, dass der Verbindung oder einem ihrer Derivate im Stoffwechselgeschehen eine bestimmte Regelfunktion zukommt, wobei das jeweilige Niveau

durch die derzeitige Stoffwechsellage bestimmt sein kann, wie dies z.B. bei Vitamin A-Mangel oder bei Thyroxinzufuhr der Fall ist.

Inwieweit es sich bei dem für Stärke resp. Fructose beobachteten Effekt um das Vorhandensein einer biologisch wirksamen Vorstufe handelt, wird durch weitere Versuche abzuklären sein.

SUMMARY

Rats were fed different diets and the ubiquinone content in the liver of these animals was investigated by means of a specific reaction. It was found that the normal ubiquinone content in rat liver is very constant, lying between 120 to 180 $\mu\text{g/g}$ liver tissue for diets containing saccharose or glucose as source of carbohydrates, and casein, lactalbumin or soya- α -protein as source of proteins. Contrary to these findings, the ubiquinone content of the livers of rats fed a diet with starch or fructose instead of saccharose or glucose is significantly higher and lies between 130 and 240 $\mu\text{g/g}$ liver tissue.

LITERATURANGABEN

- (1) Gloor, U., Schindler, O., und Wiss, O., *Helv. Chim. Acta*, **43**, 2089 (1960)
- (2) Wiss, O., Gloor, U., und Weber, F., *Amer. J. Clin. Nutr.*, in press.
- (3) Schultze, M. O., *J. Nutr.*, **61**, 585 (1957)
- (4) Kofler, M., Langemann, A., Rüegg, R., Chopard-dit-Jean, L. H., Rayroud, A., und Isler, O., *Helv. Chim. Acta*, **42**, 1283 (1959)
- (5) Shunk, C. H., Linn, B. O., Wong, E. L., Wittreich, P. E., Robinson, F. M., und Folkers, K., *J. Am. Chem. Soc.*, **80**, 4753 (1958)
- (6) Diplock, A. T., Edwin, E. E., Green, J., Bunyan, J., und Marcinkiewicz, S., *Nature*, **186**, 554 (1960)
- (7) Portman, O. W., Eleanor Y. Lawy, und Dorothy Bruno, *Proc. Soc. exptl. Biol. Med.*, **91**, 321 (1956)
- (8) Moore, T., und Sharman, I. M., *Brit. J. Nutr.*, **14**, 473 (1960)
- (9) Green, J., Edwin, E. E., Diplock, A. T., und Bunyan, J., *Biochem. Biophys. Res. Comm.*, **2**, 388 (1960)
- (10) Aiyar, A. S., Sulebele, G. A., Rege, D. V., und Sreenivasan, A., *Nature*, **184**, 1867 (1959)
- (11) Morton, R. A., und Phillips, W. E. J., *Biochem. J.*, **73**, No. 3, 427 (1959)

- (12) Moore, T., *Nature*, **184**, 607 (1959)
- (13) Edwin, E. E., Green, J., Diplock, A. T., und Bunyan, J., *Nature*, **186**, 725 (1960)
- (14) Morton, R. A., und Phillips, W. E. J., *Biochem. J.*, **73**, 421 (1959)
- (15) Olson, R. E., und Dinning, J. S., *Ann. N. Y. Acad. Sci.*, **57**, 889 (1954)

Enzymic Studies on TPN L-Hexonate Dehydrogenase from Rat Liver

By YOSHITAKE MANO, KANTARO SUZUKI, KAZUO YAMADA
and NORIO SHIMAZONO

(From Department of Biochemistry, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, February 23, 1961)

Little has been known about the reduction of uronic acid to the corresponding aldonic acid. A few examples have been presented of the reduction of hexuronic acids, *viz.* the reduction of D-glucuronic acid or its lactone by TPNH* (1-4) and that of some keturonic acids by DPNH (5-8).

On the other hand, it has been established by several workers that the immediate precursor of L-ascorbic acid in animal tissue is L-gulonolactone (9-19). However, the mechanism of formation of the lactone has not yet been elucidated completely. Previously UL Hassan and Lehninger (1) showed the reduction of D-glucuronate or the corresponding lactone by TPNH with crude rat liver extract; they considered that the enzyme might react with D-glucuronate and that the effectiveness of D-glucuronolactone might be due to the intermediary formation of D-glucuronate through the action of lactonase. Ishikawa and Noguchi (3) in this laboratory observed that D-glucuronate was formed from L-gulonate by the reverse reaction of this enzyme. The reduction of D-glucuronolactone was also observed in the reaction catalyzed by aldose reductase from sheep seminal vesicle (2), and Hers suggested the direct conversion of D-glucuronolactone to L-gulonolactone without taking into consideration on the action of lactonase (20).

In the course of the studies on the formation of ascorbic acid, Yamada (21) and

Winkelman and Lehninger (22) distinguished their lactonase into two different types, soluble lactonase and microsomal lactonase. Yamada *et al.* (15) suggested that the soluble lactonase might participate in the synthesis of L-ascorbic acid through lactonization of L-gulonate to form L-gulonolactone or of D-glucuronate to form D-glucuronolactone. Later, such reactions were actually demonstrated independently by Yamada (23), Bublitz *et al.* (17) and Chatterjee *et al.* (18).

Hence, two possibilities were considered for the formation of L-gulonolactone from D-glucuronate.

Payne and McRorie (5) and Kilgore and Starr (6) showed with bacterial enzymes the possible intermediate formation of keturonate in the conversion of D-glucuronate and D-galacturonate to L-gulonate and D-galactonate. Moreover, Ashwell (24) reported the formation of L-xylulose from D-galacturonate in the presence of TPNH by rat liver preparation *via* probable intermediates, L-galactonate and 3-keto-L-gulonate.

The present paper deals with the purification and properties of an enzyme from rat liver which oxidizes L-gulonate to D-glucuronate and discusses the position of the enzyme at the bifurcation between the pathway for L-ascorbic acid synthesis and the so-called glucuronic acid cycle, and especially its significance in the formation of L-gulonolactone. In this study, the pattern of the substrate specificity showed the validity of the name of TPN L-hexonate dehydrogenase for the enzyme rather than the names used so far, *viz.* TPN L-gulonic dehydrogenase (3, 25),

* The following abbreviations are used: Diphosphopyridine and triphosphopyridine nucleotide, DPN and TPN; their reduce form, DPNH and TPNH; Diethylaminoethyl cellulose, DEAE-cellulose; Tris (hydroxymethylamino)methane, Tris.

aldehyde reductase proposed recently by Hers (4) or glucuronate reductase (26). A part of this paper was reported previously (25, 27).

METHODS AND MATERIALS

Materials—D-Glucurono- γ -lactone, sodium D-glucuronate, ethyl D-glucuronate, and D-glucuronamide were obtained from the Chugai Pharmaceutical Company. D-Galacturonate was purchased from the Nutritional Biochemicals Corporation, D-mannurono- γ -lactone was provided through the courtesy of Dr. H. S. Isbell of the National Bureau of Standards, U.S.A., and methyl D-galacturonate was prepared by the method of Jansen and Jang (28). Methyl D-glucuronate and methyl L-gulonate were synthesized by application of the method of Ishidate *et al.* (29) in the preparation of methyl [aniline(2,3,4-tri-O-acetyl-N-glucopyranosyl)]uronate using Amberlite IR-4B and methanol. L-Idurono- γ -lactone was prepared by the method of Shafizadeh *et al.* (30), and D-xyliuronate was prepared from monoacetone D-xyliuronate, which was kindly supplied by Dr. T. Watabe of the Tokyo College of Pharmacy. L-Arabiuronate was synthesized by the method of Machida (31) from D-mannonolactone, and L-lyxuronate was the metabolic product of *Acetobacter melanogenum* (32), which was given by Dr. M. Ameyama of the Shizuoka University. L-Threuronate was prepared by the treatment with baryta for two hours at room temperature from methyl diacetyl L-threuronate, which was synthesized by the method of Lucas and Baumgarten (33). DL-Tartronic semialdehyde was furnished through the courtesy of Dr. K. Kuratomi of the Juntendo University. Malonic semialdehyde was prepared by acid hydrolysis of diethoxypropionate, which was supplied by Dr. M. Tatibana of the University of Kyoto, and succinic semialdehyde was synthesized by decarboxylation of formylsuccinic acid diethylester, which was synthesized by the method of Wislicenus *et al.* (34). Glutaric semialdehyde was synthesized from cyclopentanol by the method of Harries and Tank (35). Methylformyl acetate was prepared by the method of Wislicenus (36). D-Fructuronate was kindly supplied by Dr. G. Ashwell of the National Institutes of Health, U.S.A. and D-tagaturonate was given by Dr. Y. Takagi of the Kanazawa University. 2-Keto-L-gulonate and its methyl ester were obtained from the Takeda Chemical Industries. 2-Keto- and 5-keto-D-gluconate were provided through the courtesy of Dr. R. Takeda of the Takeda Chemical Industries. Keto-DL-erythronate* and DL-

erythronate were prepared by Neuberg's method (37), through oxidation of *meso*-erythritol with nitric acid. Hydroxypyruvate was kindly supplied by Dr. K. Kuratomi of the Juntendo University.

D-Glucosone was synthesized from D-glucose according to the method of Becker and Day (38). D-Galactosone, D-xylosone, and L-arabinosone were also prepared by a modification of the above method. Methylglyoxal was prepared by the oxidation of acetone with selenium dioxide (39). Glyoxylate, glyoxal, ethylene glycol, pyruvate, α -ketobutyrate, acetoacetate and β -hydroxybutyrate were commercial products of the Tokyo Kasei Company.

Acetaldehyde was freshly prepared from paraldehyde with trace of sulfuric acid. *n*-Propion-, *n*-butyr-, *n*-valer-, croton-aldehyde, aldol and acrolein were purchased from the Tokyo Kasei Company. DL-Lactic aldehyde was obtained by hydrolysis of lactic aldehyde acetal, which was synthesized from methylglyoxal (40). Hydracryl aldehyde was prepared from acrolein by the method of Nef (41). Malonic, succinic and glutaric dialdehyde were synthesized from β -ethoxyacroleinacetal, succinodialdoxim and cyclopentenozonide by the method of Claisen (42), Harries (43), and Harries and Tank (35), respectively.

D- and L-Glyceraldehyde were prepared from acetone D-glyceraldehyde, which was synthesized by Baer and Fisher (44), and from L-arabinose (45). DL-Glyceraldehyde was obtained from the Nutritional Biochemicals Corporation, and its 3-phosphate was the gift from Dr. K. Nagasawa of the University of Tokyo. D-Erythrose was kindly supplied by Dr. K. Uehara of the University of Osaka, and D-threose was obtained by Ruff's degradation from strontium D-xylonate (46). L-Galactose was that prepared by Dr. C. Araki of the Kyoto University of Industrial Arts and Textile Fibers. D-Ribulose was kindly provided by Dr. I. Yanagisawa of the Toho University, and L-xylulose was prepared from L-xylose by isomerization with pyridine (47). 2,3,4,5,6-Pentaacetyl D-glucose and 2,3,4,6-tetraacetyl D-glucose were synthesized by the methods of Wolfrom (48) and of Fischer and Hess (49), respectively. 1,2-O-Isopropylidene-D-xylo-dialdopentofuranose was synthesized by the method of Schaffer and Isbell

* Keto-DL-erythronate used here corresponds to "Oxyerythronsäure" named by C. Neuberg (37). The compound may be thought as keturonate rather than undefined structure because of no consumption of iodine in the determination by Willstätter and Schudel's method (66), but the position of the keto group has not been clarified.

(50), and it was then converted to D-xylo-dialdopentofuranose by the treatment with 0.5 *N* sulfuric acid at 50°C for 2 hours. Other sugars and their derivatives were obtained as commercial products.

L-Gulonon- γ -lactone was prepared by the reduction of D-glucuronate with sodium borohydrate according to the method of Wolfrom and Anno (51), and L-galactono- γ -lactone was synthesized by a similar method as described by Mapson and Breslow (52). L-Mannono- γ -lactone was prepared from L-arabinose by the modification of the Kiliani synthesis as described by Isbell *et al.* (53), and D-mannono- γ -lactone was synthesized by the oxidation of D-mannose according to the method of Hudson and Isbell (54); the latter was also provided by Dr. H. S. Isbell and Dr. G. Ashwell. D- and L-Glucono- δ -lactone were synthesized by the method of Isbell *et al.* (53) from D- and L-arabinose, and D-glucono- γ -lactone was prepared by heating from D-gluconate. D-Allono- and D-altrono- γ -lactone were synthesized by the method of Phelps and Bates (55). D-Idono- and D-talono- γ -lactone were prepared by the isomerization of D-gulono- and D-galactono- γ -lactone with pyridine according to the method of Fischer and Fay (56) and Glatthaar and Reichstein (57), respectively. The latter was also kindly provided by Dr. H. S. Isbell. L-Idonate was kindly provided by Dr. Y. Takagi of the University of Tokyo. D-Gulono- and D-galactono- γ -lactone were purchased from the Nutritional Biochemicals Corporation. L-Rhamnono- γ -lactone was obtained by oxidation of L-rhamnose by bromine (58). D- α - and β -Glucoseptono- γ -lactone were synthesized by the method of Hudson (59) using methylcellosolve in their differential crystallization. D-Xylono-, L-xylono-, D-arabono-, L-arabono-, D-lyxono- and L-lyxono- γ -lactone were synthesized by an application of the method of Clowes and Tollens (60). D-Ribono- γ -lactone was obtained from the Nutritional Biochemicals Corporation. L-Threono- γ -lactone was prepared by the degradation of L-ascorbic acid according to the method of Hardegger *et al.* (61). DL-Glycerate was synthesized by oxidation of DL-glyceraldehyde according to the method of Wohl and Schellenberg (62). β -Hydroxypropionate and acetol were prepared by the method of Wislicenus (63) and Levene and Walti (64), respectively. N-Acetylneuraminate was kindly supplied by Dr. T. Yamakawa of the University of Tokyo, and glycolate was obtained from the Tokyo Kasei Company. Free acids of various aldonic acids described above were obtained by hydrolysis of the corresponding lactones with an equivalent amount of sodium hydroxide in hot water (60°C), and reversely, some lactones were

prepared from the corresponding acids by heating (ca. 70°C) at pH about 3.

L-Gulonamide was synthesized as follows: 3 g. of L-gulonolactone was suspended in 9 ml. of methanol, and 3 ml. of 28 per cent ammonia water was added gradually under continuous stirring at room temperature. After the addition the stirring was continued for about 30 minutes, and then the clear mixture was evaporated under reduced pressure with repeated addition of methanol. The resulting syrup was made to crystallize by the addition of methanol. The product decomposed at 126–128°C.

D-Sorbitol, D-mannitol, *meso*-dulcitol, *meso*-adonitol, and *meso*-erythritol were obtained as commercial products. L-Threitol was synthesized according to the method of Lucas and Baumgarten (33).

TPN and DPN were obtained from the Sigma Chemical Company, and their reduced forms were prepared chemically with dithionite (65). In some experiments for the coupling reaction with L-gulonolactone dehydrogenase, pure β -TPNH preparation obtained from Sigma Chemical Company was used. Isocitrate was purchased from the Nutritional Biochemicals Corporation.

Lycorine hydrochloride was kindly supplied from Dr. K. Yamamoto of the Shionogi Research Laboratory. Other chemicals were furnished as commercial products. DEAE-Cellulose was synthesized from 2-chlorotriethylamine hydrochloride and cellulose (100 mesh, Toyo Roshi Co., Ltd.) by the method of Peterson and Sober (67).

Enzymes—L-Gulonolactone dehydrogenase was prepared from rat liver as described in a previous paper (68). Isocitric dehydrogenase was obtained by the method of Ochoa (69).

Assay Method—The assay of the enzymic reaction was carried out by the determination of the change in optical density at 340 $m\mu$ due to the oxidation of TPNH or the reduction of TPN using the Hitachi spectrophotometer equipped with cells having 1 cm. light path. The standard reaction mixture contained 50 μ moles of phosphate buffer of pH 7.0, 0.5 μ mole of TPN or TPNH, 10 μ moles of substrate (3.3×10^{-3} M), and 30 μ g. of the purified enzyme. The total volume of the system was made to 3.0 ml. Prior to the start of reaction, all components except substrate were kept in a water bath of 37°C for 5 minutes, and the reaction was started by addition of the substrate in the same bath, and the determination was completed within 10 minutes. The unit of activity of the enzyme was defined as the change of optical density at 340 $m\mu$ in 10 minutes $\times 10^3$, and the specific activity of the enzyme was expressed as units per mg. of protein. Unless otherwise stated, the reaction was

carried out in the direction of reduction of the substrate (D-glucurono- γ -lactone was the usual substrate). In experiments with particulate preparations or homogenates, determination was carried out by the opal glass technique (70). In the case of crude preparation, 30 μ moles of nicotinamide was added to inhibit diphosphopyridine nucleotidase. Both reactions, dehydrogenation and reduction, could proceed only in the complete system, and any other non-enzymic process could not be detected by this assay method.

Determination of D-Glucurono- γ -lactone, D-Glucuronate and L-Gulonolactone—D-Glucurono- γ -lactone and D-glucuronate were estimated by the method of Ishidate and Nambara (71). Determination of L-gulonolactone was carried out using microsomal L-gulonolactone dehydrogenase (68) as follows. To the reaction mixture to be estimated, in this case, sodium 5, 5-diethylbarbiturate was added to a final concentration of $10^{-2} M$ to stop the reaction specifically (see later), and the aliquot of the mixture was taken for determination. Microsomes prepared by the method of Schneider and Hogeboom (72) from 0.8 g. of rat liver and washed three times, were added to the sample to be determined. The mixture was shaken in an oxygen phase at 37°C for 30 minutes. After the reaction, the mixture was deproteinized with an equal volume of 20% trichloroacetic acid and the ascorbic acid formed was determined by the method of Roe and Kuether (73). The quantity of L-gulonolactone formed was calculated by comparison with a standard curve. The optimal concentration range of L-gulonolactone for the measurement is 10–60 μ g./3 ml., and the method is fairly specific for the determination of L-gulonolactone and L-galactonolactone with a few exceptions (68).

RESULTS

Purification of the Enzyme—Rats weighing about 150–200 g. were killed by decapitation under ether anaesthesia, and livers* from 10 rats were minced and homogenized with 4 volumes of 0.25 M sucrose using the Potter-Elvehjem glass homogenizer. The homogenate was immediately subjected to centrifugation at 105,000 $\times g$ for 60 minutes at 0°C. The resulting precipitate was discarded and

a clear supernatant (about 300 ml.) was pooled. All the subsequent steps were carried out at 0° to 5°C.

To each 100 ml. of the above supernatant, 23 g. of solid ammonium sulfate was added, giving a final saturation of 0.38. After standing for about 2 hours the mixture was centrifuged and the precipitate was discarded. To the supernatant, ammonium sulfate was further added (12.2 g. to each 100 ml. of the original extract) to a saturation of 0.55. The solution was again centrifuged after standing for 2 hours and the supernatant was discarded. The precipitate was dissolved in 300 ml. of water and 72.9 g. of ammonium sulfate was added to the solution (about 0.40 saturation), and after centrifugation another portion of ammonium sulfate (20.2 g.) was added to the supernatant to reach the saturation of 0.50, and allowed to stand for 2 hours. The precipitate resulting after centrifugation of this material was dissolved in 300 ml. of 0.40 saturated ammonium sulfate solution and to the supernatant another portion of ammonium sulfate (18.9 g.) was added to reach the saturation of 0.50. The above two solutions saturated in 0.50 with ammonium sulfate were spun down and the precipitates were combined. The precipitate was dissolved in a minimum amount of water (about 3 ml.) and the enzyme solution was dialyzed against about 2 liters of distilled water until the trace of sulfate ions of the dialysate could not be detected by barium acetate. The resulting insoluble matter was spun down and discarded. The clear solution was diluted with water and Tris buffer (pH 7.5) until its protein concentration became below 0.2 per cent and the final concentration of Tris buffer became 0.02 M , and then the solution was subsequently subjected to DEAE-cellulose column chromatography. DEAE-cellulose used for the chromatography was treated with N NaOH and N HCl and buffered with 0.02 M Tris buffer of pH 7.50. The diluted enzyme solution was passed through a DEAE-cellulose column (2 \times 5 cm.) with pumping by means of suitably controlled air pressure, at a flow rate of 10–15 ml. per 10

* Although kidney, as described later, showed the highest activity regarding this enzyme, liver was chosen as the enzyme source because it is the only organ that can synthesize L-ascorbic acid in rats (100).

minutes, and each effluent (5 ml.) was collected by a fraction collector. The column was then washed with about 200 ml. of the same buffer, and the enzyme was eluted usually as the second peak in this fraction (tube number, about 30) (Fig. 1). However, the position of this second peak was somewhat variable, probably influenced by the exchange capacity. Each active fraction was collected and after the pH was adjusted to 7.0, the enzyme could be stored in a frozen state, where it was fairly stable for 3 weeks at around -20°C . The summary of the purification is presented in Table I. The specific activity of the purified preparation was about 500 times that of the crude extract.

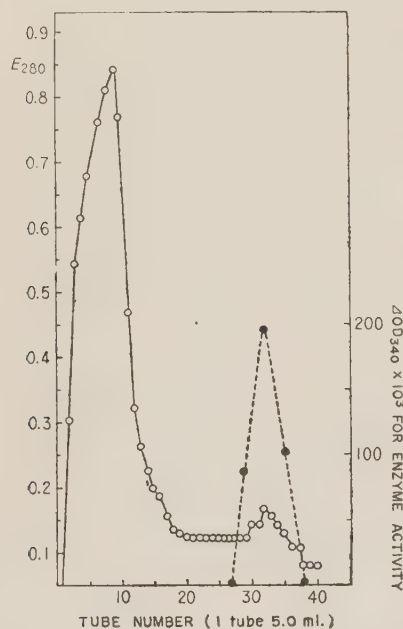


FIG. 1. Chromatographic purification of the enzyme on DEAE-cellulose column.

—○— Absorption at $280\text{ m}\mu$ of the eluate,
 --●-- Enzyme activity.

The chart of purification with the oxidation of L-galactonate by TPN as an indicator of the activity showed a precise agreement with that obtained in the reduction of D-glucurono- γ -lactone by TPNH described above. This fact may indicate that the

enzyme participating in these two reactions is the same.

TABLE I
 Summary of Purification Procedure

	Total volume (ml.)	Total units	Total protein (mg.)	Specific activity (units per mg.)	Recovery (%)
Extract	300	29700	5589	5.3	100
Ammonium sulfate fractionation	45.2	19090	266.7	71.6	64
DEAE-cellulose treatment	40.0	13480	5.3	2543	45

Formation of L-Gulono- γ -lactone from D-Glucurono- γ -lactone (25)—The enzyme catalyzed the reduction of D-glucuronolactone as well as D-glucuronate by TPNH in spite of the complete absence of the lactonase. On measurement by the method of Yamada (21), spontaneous cleavage of the lactone to its acid under the reaction condition was shown to be negligible. The reaction product from D-glucuronolactone was identified as L-gulonolactone by means of paper chromatography with three different solvent systems detected by periodate consumption (74) and hydroxamate formation (75) (Table VI). In the reaction mixture the TPNH-regenerating system with isocitrate and isocitric dehydrogenase was added. Under the condition in which the reaction was carried out, L-gulonolactone could by no means be formed from L-gulonate enzymatically (23). To detect the formation of L-gulonolactone, L-gulonolactone dehydrogenase was added and the formation of L-ascorbic acid from D-glucuronolactone was shown in this coupled system (see next section). From the above results it was concluded that the direct conversion of D-glucuronolactone to L-gulonolactone must have occurred in this reaction, and this was supported also by the following evidences: 1) the initial velocity of reaction of the lactone was greater than that of the acid in concentrations over $1.54 \times 10^{-3} M$ (Fig. 2); 2) the ratio of activity of the lactone to that of the acid was constantly 1.2 for each step of

the purification (Table II); 3) the ratio of activity to two substrates was in good agreement in each effluent of DEAE-cellulose column

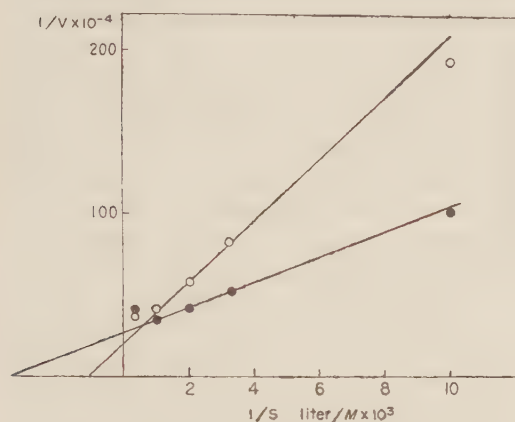


FIG. 2. Influence of concentration on activity with D-glucuronate and D-glucuronolactone. —○— D-Glucuronolactone, —●— D-Glucuronate.

chromatography (Table II); 4) various animal organs lacking lactonase I (soluble lactonase) activity, for example, human liver, guinea pig

TABLE II

Constant Activity Ratio of Reduction of D-Glucuronolactone to D-Glucuronate at Each Purification Step

	Extract	Ammonium sulfate fractionation	DEAE-cellulose treatment
D-Glucuronolactone	84	186	497
D-Glucuronate	70	145	410
Ratio (lactone: acid)	1.20	1.28	1.21

Constant Activity Ratio to Two Substrates in Each Effluent from DEAE-Cellulose Column

Tube number	30	32	34	36	38
D-Glucuronolactone	282	511	360	180	60
D-Glucuronate	230	418	303	147	51
Ratio (lactone: acid)	1.23	1.22	1.19	1.21	1.18

liver and rat kidneys (15), were found to be active with D-glucuronolactone (Table VIII); and 5) the latter was active as substrate in the presence of $10^{-4}M$ *p*-chloromercuribenzoate

which inhibits the lactonase completely (21) (Table IX). The Michaelis constants for D-glucuronolactone and D-glucuronate were $6.9 \times 10^{-4}M$ and $3.3 \times 10^{-4}M$, respectively, and the Lineweaver-Burk's plots (76) (Fig. 2) for these substrates crossed each other near the point corresponding to $S = 1.54 \times 10^{-3}M$, and the fact also suggests that the conversion is the direct one.

Stoichiometry and Equilibrium Constant—

Stoichiometry of the reaction at equilibrium was found by the chemical determination of D-glucuronolactone consumed and TPNH oxidized compared with the enzymic assay of L-gulonolactone produced and TPN formed. From the data listed in Table III, the equilibrium constant of the reaction was calculated to be 3.2×10^{-2} ; in regard to the limiting factor, TPNH, 89 per cent of TPN and 11 per cent of TPNH were present at equilibrium. Thus the equilibrium is far to the side of reduction of the substrate, *i.e.* the formation of L-gulonolactone. In a similar way, an attempt was made to determine the equilibrium constant for the system of D-glucuronate, and the value of 2.6×10^{-2} (75 per cent of TPN and 25 per cent of TPNH) was obtained by calculation from the data of D-glucuronate consumed and TPNH oxidized assuming that the stoichiometry of the reaction was established. The result shows a little less inclination of the reaction toward reduction in the formation of L-gulonate than in the corresponding formation of L-gulonolactone. As will be seen later, the stoichiometric relation between ethyl D-glucuronate consumed and L-gulonolactone formed could be observed (*cf.* Table VII). The establishment of the stoichiometry may show the direct conversion of uronate to aldinate or of uronolactone to aldinate without the intermediary accumulation of any keturonate.

Substrate Specificity: Reduction Process—The enzyme catalyzes the reduction of other derivatives of various sugars by TPNH, which could not be replaced by DPNH. The broad specificity manifested even by highly purified preparation is characteristic of the reduction process in contrast to the strict specificity of

the dehydrogenation process (Table IV).

The enzyme acted on alduronic acids of which the carbon number varies from six to three; D-glucuronate, D-galacturonate, D-mannuronate, L-iduronate, L-threuronate, and DL-

TABLE III
Stoichiometry of the Reaction¹⁾

	D-Glucurono- lactone	TPNH	L-Gulono- lactone	TPN ²⁾
	μmole	μmole	μmole	μmole
Initial state	10.0	0.44	0	0
Final state	9.62	0.05	0.37	0.39
Difference	-0.38	-0.39	+0.37	+0.39

1) Reaction period, 60 minutes.

2) The quantity of TPN formed was calculated from the spectral difference at 340 m μ , assuming the difference of molecular extinction between oxidized and reduced forms is 3.0×10^3 .

tartronic semialdehyde, with the exception of five, D-xyliuronate, D-lyxuronate, and L-arabiuronate. However, it did not act on the corresponding keturonates, such as D-fructuronate, D-tagaturonate, 2-keto-L-gulonate and its methyl ester, 2-keto- and 5-keto-D-gluconate, keto-DL-erythronate, and hydroxypyruvate, and other keto acids such as α -keto-butyrate, pyruvate and acetoacetate. The same results were also obtained in experiments with crude homogenates. The enzyme also catalyzed the reduction of such derivatives as lactones, esters, and an amide of the above-mentioned alduronic acids, *viz.* D-glucurono- γ -lactone, ethyl D-glucuronate, D-glucuronamide, methyl D-galacturonate, D-mannurono- γ -lactone, L-idurono- γ -lactone, and methyl diacetyl L-threuronate; under the conditions studied, the activities toward lactones were higher and toward esters were lower, in general, than the corresponding acids, respectively. The enzyme proved to be also active on glyoxylate, malonic semialdehyde, succinic semialdehyde, and glutaric semialdehyde, all of which may be regarded as a sort of uronate. Methylformyl acetate, a branched semialdehyde, was also effective as substrate.

Osones derived from hexose, D-glucosone

and D-galactosone, were accessible, whereas those from pentose, D-xylosone and L-arabinoxosone, were not. Methylglyoxal was proved to be the most active among the compounds tested.

Of various aldomonosaccharides examined, those with carbon number of three and four, D- and L-glyceraldehyde, D-erythrose, D-threose, and DL-glyceraldehyde-3-phosphate, were reactive, while those with carbon number from five to six, D- and L-lyxose, D- and L-arabinose, D- and L-xylose, D-glucose, D- and L-galactose, D-mannose, L-rhamnose, L-fucose, D-glucose- and D-galactosamine, were not, with the exception of D-ribose which showed a slight activity. Phosphorylated sugars, D-glucose- and D-galactose-6-phosphate showed a little activity. Glycolaldehyde was also reactive. Various ketomonosaccharides, sedoheptulose, L-sorbose, D-fructose, D-tagatose, D-ribulose, L-xylulose and dihydroxyacetone, did not serve as substrate.

The enzyme was very active with dialdehydes, *viz.* glyoxal, malonic dialdehyde, succinic dialdehyde and glutaric dialdehyde. However, these activities may indicate a two step reduction to the alcohol, *i. e.* dialdehyde \rightarrow monoaldehyde \rightarrow alcohol, since the corresponding monoaldehydes have been proved to be also effective as substrate of the enzyme. D-Xylodialdo-pentofuranose was also accessible. Typical aldehydes, such as acet-, propion-, *n*-butyr-, and *n*-valeraldehyde, were somewhat reactive; the activities were approximately parallel with the chain length. Other types of aldehyde, *viz.* benzaldehyde, DL-lactic aldehyde, hydracryl aldehyde, aldol, and acrolein were also reducible.

This pattern of specificity shows that there is no definite relation between activity and configuration. These observations suggest that the enzyme attacks the carbohydrate in oxo-form and other free aldehyde compounds, except those derived from pentose, in other words, a free aldehyde group is essential for the activity. The activity for uronate may be considered to be of special case. That the oxo-form is apparently the true substrate for the enzyme was further confirmed by the following experiments. 2, 3, 4, 5, 6-Penta-acetyl

D-glucose was effective as substrate, whereas 2, 3, 4, 6-tetraacetyl D-glucose was ineffective. Furthermore, the activities observed with some sugars, D-ribose, L-arabinose, D-galactose, and D-glucose at a higher concentration, $3.3 \times 10^{-2} M$, were in good correlation to the contents of oxo-form.* The conclusion was also supported by the formation of D-xylose from D-xyloaldopentofuranose as shown later.

The Michaelis constant of the enzyme

was determined for some representative compounds in their reduction according to Lineweaver and Burk (76). K_m for D-glucuronolactone, D-glucuronate, D-galacturonate, and DL-glyceraldehyde were estimated as $6.9 \times 10^{-4} M$, $3.3 \times 10^{-4} M$, $9.1 \times 10^{-5} M$ and $1.54 \times 10^{-3} M$, respectively.

Dehydrogenation Process—In contrast to the broad substrate specificity in the reduction process, the enzyme showed a narrow specificity in the dehydrogenation process (Table

TABLE IV
Substrate Specificity of TPN L-Hexonate Dehydrogenase in Reduction process

Substrate	Activity (unit)	Substrate	Activity (unit)
D-Glucuronate	355	Acrolein	37
D-Glucurono- γ -lactone	420	DL-Lactic aldehyde	465
Ethyl D-Glucuronate	327	Hydracryl aldehyde	620
D-Glucuronamide	138	D-Glucosone	405
D-Galacturonate	449	D-Galactosone	488
Methyl D-Galacturonate	370	D-Xylosone	11
D-Mannuronate	414	L-Arabinosone	26
D-Mannurono- γ -lactone	445	Methylglyoxal	974
L-Iduronate	524	D-Glucose	0 (0)
L-Idurono- γ -lactone	580	D-Galactose	0 (10)
D-Xyliurionate	0	D-Mannose	0
D-Lyxurionate	12	L-Galactose	0
L-Arabiurionate	17	D-Ribose	54 (174)
L-Threuronate	280	D-Arabinose	0
Methyldiacetyl L-threuronate	728	D-Xylose	0
DL-Tartronic semialdehyde	332	D-Lyxose	0
Glyoxylate	556	L-Arabinose	0 (38)
Glutaric semialdehyde	380	L-Xylose	0
Succinic semialdehyde	625	L-Lyxose	0
Malonic semialdehyde	460	D-Erythrose	412
Methylformyl acetate	510	D-Threose	172
D-Fructuronate	24	D-Glyceraldehyde	484
D-Tagaturonate	15	L-Glyceraldehyde	592
2-Keto-L-gulonate	18	DL-Glyceraldehyde	903
Methyl 2-keto-L-gulonate	27	Glycolaldehyde	119
2-Keto-D-gluconate	8	L-Rhamnose	0
5-Keto-D-gluconate	26	L-Fucose	0
Keto-DL-erythronate	15	D-Xyloaldopentofuranose	446
Hydroxypyruvate	8	2, 3, 4, 5, 6-Pentaacetyl D-glucose	340
α -Ketobutyrate	0	2, 3, 4, 6-Tetraacetyl D-glucose	8
Pyruvate	0	Glucose-6-phosphate	80
Acetoacetate	0	Galactose-6-phosphate	47
Glutaric dialdehyde	585	DL-Glyceraldehyde-3-phosphate	115
Succinic dialdehyde	520	Sedoheptulose	0
Malonic dialdehyde	625	D-Sorbose	0
Glyoxal	651	D-Fructose	0
Benzaldehyde	446	D-Ribulose	0
n-Valeraldehyde	433	L-Xylulose	0
n-Butyraldehyde	123	Dihydroxyacetone	0
Propionaldehyde	49	Acetol	0
Acetaldehyde	16	D-Glucosamine	0
Crotonaldehyde	61	D-Galactosamine	0
Aldol	118		

() indicates the activity when substrate was used at $3.3 \times 10^{-2} M$.

* Compared with the data of Cantor, S. M., and Peniston, Q. P., *J. Am. Chem. Soc.*, **62**, 2113 (1940)

TABLE V

Substrate Specificity of TPN L-Hexonate Dehydrogenase in Dehydrogenation Process

Substrate	Activity (units)	
	Acid	Lactone (γ)
D- α -Glucoheptonic	0 (0)	0 (0)
D- β -Glucoheptonic	0 (0)	0 (0)
D-Allonic	0 (0)	0 (0)
D-Altronic	0 (0)	0 (0)
D-Gluconic	0 (0)	0 (0) [0]
D-Mannonic	68 (18.7)	40 (11.0)
D-Gulonic	0 (0)	0 (0)
D-Idonic	0 (0)	0 (0)
D-Galactonic	0 (0)	0 (0)
D-Talonic	0 (0)	0 (0)
L-Gluconic	147 (41)	— [128(35.4)]
L-Mannonic	251 (69.5)	109 (30)
L-Gulonic	362 (100)	168 (46.5)
L-Idonic	397 (109)	226 (62.5)
L-Galactonic	177 (49)	87 (24)
L-Rhammonic	0 (0)	0 (0)
D-Ribonic	0 (0)	0 (0)
D-Arabonic	0 (0)	0 (0)
D-Xylonic	0 (0)	0 (0)
D-Lyxonic	0 (0)	0 (0)
L-Arabonic	0 (0)	0 (0)
L-Xylonic	0 (0)	0 (0)
L-Lyxonic	0 (0)	0 (0)
DL-Erythronic	0 (0)	—
L-Threonic	0 (0)	0 (0)
DL-Glycerate	0 (0)	—
N-Acetyl neuraminate	0 (0)	—
β -Hydroxypropionate	0 (0)	—
Glycolate	0 (0)	—
D-Sorbitol	0 (0)	—
D-Mannitol	0 (0)	—
meso-Dulcitol	0 (0)	—
meso-Adonitol	0 (0)	—
meso-Erythritol	0 (0)	—
L-Threitol	0 (0)	—
Glycerol	0 (0)	—
Ethylene glycol	0 (0)	—
Ethanol	0 (0)	—

() shows the relative activity to that of L-gulonate as 100.

[] shows the activity to their δ -lactones.

V).

The enzyme oxidized exclusively the L-form of both hexonic acids and their lactones, namely, L-gluconate and its δ -lactone, L-mannonate, L-gulonate, and L-idonate and their γ -lactones, with an exception of minute activity of D-mannonate and its γ -lactone. In general, the activity was higher in the acid form than in the lactone form. Another exception, ineffectiveness of L-rhamnonate and its γ -lactone as substrate, would be indicative of necessity of the presence of alcohol group at C-6 for the enzyme activity.

Aldonates and their derivatives other than those described above were all ineffective : D-gluconate and its δ - and γ -lactone; D- α - and β -glucoheptonate, D-allonate, D-altronate, D-gulonate, D-idonate, D-galactonate, D-talocate, D-ribonate, D- and L-arabonate, D- and L-xylonate, D- and L-lyxonate, L-threonate, and their γ -lactones; DL-erythronate, DL-glycerate, glycolate, N-acetyl neuraminate, β -hydroxypropionate, and β -hydroxybutyrate.

Various polyalcohols, such as D-sorbitol, D-mannitol, meso-dulcitol, meso-adonitol, meso-erythritol, L-threitol, glycerol, and ethylene glycol, were also inactive. Of the probable products in the reduction process the various ketoses, which contain acetol, were not oxidized.

Thus only such reactions in which the reaction product is L-hexonate were reversible while others irreversible.

This pattern of specificity did not change at the optimum pH, 9.6–10.0. As far as were tested in animal tissues, this pattern of specificity was confirmed irrespective of the source of the enzyme.

All the facts indicate that for the dehydrogenation of hexonate the *levo* configuration of the hydroxyl group at C-5 would be essential.

Reaction Product—It was shown previously that the reaction product in the reduction of D-glucuronolactone by TPNH is L-gulonolactone. The reduction product from D-gluconate was identified as L-gulonate in the same way (Table VI). A direct transformation of ethyl D-glucuronate and methyl D-

galacturonate to each L-gulonolactone and L-galactonolactone in the reduction processes was verified in a similar manner under the condition of complete absence of esterase, showing an agreement with the findings of Mapson and Isherwood (77) in the plant system. In these experiments no inter-

mediary appearance of the corresponding esters of L-gulonate and L-galactonate was detected. The establishment of stoichiometry in the case of ethyl D-glucuronate as substrate (Table VII) would also support the direct conversion to L-gulonolactone. The reduction product from D-glucuronamide was found to

TABLE VI
Paper Chromatography of Reaction Product

	<i>n</i> -Butanol ethanol water (4:1:1)	<i>n</i> -Butanol acetic acid water (4:1:2)	Ethanol acetic acid water (8:1:1)	Acetone water (4:1)
Authentic sample	<i>R_f</i>	<i>R_f</i>	<i>R_f</i>	<i>R_f</i>
D-Glucuronate	0.03	0.24	0.24	0.14
D-Glucurono-γ-lactone	0.34	0.47	0.57	0.83
D-Glucuronamide	—	0.28	0.27	0.25
D-Galacturonate	0.06	0.29	0.33	—
Methyl D-galacturonate	0.33	0.47	0.52	—
L-Gulonate	0.09	0.37	0.27	0.19
L-Gulono-γ-lactone	0.23	0.43	0.44	0.65
L-Galactonate	0.19	—	0.31	—
L-Galactono-γ-lactone	0.33	0.46	0.39	—
Ethyl L-gulonate	0.48	—	0.53	—
Methyl L-galactonate	0.27	—	0.44	—
L-Gulonamide	0.12	0.30	0.30	0.19
D-Xylose	0.47	0.42	—	—
D-Xylodialdopentofuranose	0.58	0.60	—	—
Reaction product ¹⁾				
Substrate				
D-Glucurono-γ-lactone	0.35, <u>0.22</u> 0.10, <u>0.02</u>	—	0.58, <u>0.44</u> 0.26, <u>0.23</u>	0.84, <u>0.66</u> 0.19, <u>0.15</u>
D-Glucuronate	0.03, <u>0.08</u>	<u>0.37</u> , 0.25	0.55, <u>0.25</u>	—
Ethyl D-glucuronate	—	0.52, <u>0.42</u> 0.22, <u>0.35</u>	0.60, <u>0.42</u> 0.22, <u>0.25</u>	—
Methyl D-galacturonate	<u>0.32</u> , 0.30 0.19	—	0.51, 0.33 <u>0.40</u> , 0.30	—
D-Glucuronamide	—	0.26, <u>0.30</u> 0.22	—	—
D-Xylodialdopentofuranose	0.58, <u>0.46</u>	0.61, <u>0.43</u>	—	—

1) The value underlined indicates the *R_f* corresponding to probable product.

The reaction mixture consisted of 500 μmoles of substrate, 500 μmoles of DL-isocitrate, 10 μmoles of TPN, 500 μmoles nicotinamide, 1.25mmoles of glycylglycine buffer, pH 7.3, TPN L-hexonate dehydrogenase, and isocitric dehydrogenase in a total volume of 50 ml. was incubated at 37°C for 30 minutes. After incubation, the reaction was stopped by addition of 6 ml. of 60 % perchloric acid, and the supernatant was neutralized with KOH by use of methyl red. The precipitate was spun down and the supernatant was used as a sample after lyophilization. Ascending method; front movement, about 300 mm., Toyo filter paper No. 53.

be L-gulonamide by paper chromatography. The undefined formation of L-gulonolactone which could be detected in paper chromatogram in some cases may be thought as an artificial product, because, as will be discussed later, an artefactual lactonization of these derivatives would occur during the operation of lyophilization. The formation of L-gulonolactone from D-glucuronolactone and ethyl D-glucuronate, and of L-galactonolactone from methyl D-galacturonate was further deduced from the results of the coupling reaction with L-gulonolactone dehydrogenase leading to the formation of L-ascorbic acid.

D-Xylose was identified by paper chromatography from the reaction mixture when D-xylodialdopentofuranose was applied as substrate for the reduction reaction. The possibility that D-fructose is formed from D-glucosone and acetol from methylglyoxal was suggested through the use of the reactions of Roe *et al.* (78) and of Baudisch (79), respectively. These findings would show that the reduction is limited only to the aldehyde group.

The oxidation product of L-gulonate by TPN was demonstrated previously as D-glucuronate in our laboratory (3). As for the question of the oxidation product of L-galactonolactone, D-galacturonate was identified as the sole product and the lactone form of the uronate was not detected. The product of the oxidation of L-galactonate by TPN was demonstrated to be D-galacturonate instead of L-xylulose (*cf.* 24). The same result was obtained in the experiments with the purified preparation starting from the acetone dried powder of rat liver. Carbon dioxide evolution in the oxidation of L-galactonate by TPN was not detected manometrically.

Formation of D-fructuronate from D-glucuronolactone and D-glucuronate and of D-tagaturonate from D-galacturonate was examined by the method of Watanabe and Arai (80), but the occurrence of such reactions could not be detected at any stage of the reaction, respectively. Therefore, the re-

duction of D-glucuronolactone, D-glucuronate, and D-galacturonate to the corresponding hexonate or its lactone may proceed in one-step process.

TABLE VII

Stoichiometric Formation of L-Gulonolactone from Ethyl D-Glucuronate

Substrate reacted	L-Gulonolactone formed
0.247 μ mole	0.240 μ mole

Reaction condition: Ethyl D-glucuronate, 20 μ moles; TPNH, 1 μ mole; enzyme, 60 μ g.; phosphate buffer, 50 μ moles, pH 7.0. Total volume, 3.0 ml. Incubation, 37°C, 30 minutes. Microsomes derived from 0.89 g. of rat liver was used for the assay system.

Distribution of the Enzyme—The enzyme is found only in the "supernatant fraction" defined in the fractionation of cellular components after Schneider and Hogeboom (72). The supernatant fractions derived from various organs of rat contained this enzyme (Table VIII). Among the organs tested kidney showed the highest specific activity. In heart, brain, and spleen, the activity of the enzyme was not detected except for glyceraldehyde and glucosone. The pattern of specificity of these organs was not so different from each other as was suggested by Hers (4, 20). Human liver and both liver and kidney from guinea pig and rabbit showed a considerable activity with two substrates, D-glucuronolactone and D-glucuronate. Some of avian tissues also contained this enzyme. These facts show that the distribution of the enzyme in organs or species has no definite relation with the L-ascorbic acid synthesizing activity.

Activation and Inactivation—The enzyme was activated by sulfate ions to about 150 per cent at the concentrations of 10^{-2} M or more. None of other compounds tested could accelerate the reaction (Table IX). Apparent stimulation by Mn^{++} and Co^{++} was disclosed as non-enzymic nature. Hg^{++} and Cu^{++} strongly inhibited the enzyme at the concentrations of 10^{-4} M or less. On the other hand, *p*-chloromercuribenzoate hardly in-

TABLE VIII

Distribution of the Enzyme in Various Organs of Rat

Substrate	D-Glucuronic acid	D-Glucurono-lactone	D-Galacturonic acid	D-Glucose	D-Glucosone	DL-Glycer-aldehyde
Liver	16.8	21.4	16.2	0	20.5	94.6
Kidney	83.2	131.0	98.3	0	128.7	274.0
Skeletal muscle	21.6	42.3	26.8	0	32.0	72.3
Seminal vesicle	15.3	37.0	45.2	0	48.2	91.0
Testis	23.3	46.5	18.0	0	46.4	107.2
Heart	0	0	3.8	0	31.2	38.3
Brain	0	0	0	0	32.8	19.5
Spleen	0	2.9	0	0	16.7	13.8

Values are presented in specific activity (units/mg. protein)

TABLE IX

Effect of Some Compounds Which Affect the Metabolism of Ascorbic Acid on the Enzyme

Compounds	Concentra-tions added (M)	Inhibition or activation (per cent)
Chloretone	3.3×10^{-3}	- 8
Barbital (5, 5-Diethylbar-bituric acid)	3.3×10^{-3}	-92
Antipyrine	3.3×10^{-3}	0
4-Aminoantipyrine	3.3×10^{-3}	0
Lycorine hydrochloride	3.3×10^{-3}	+6
p-Chloromercuribenzoate	1×10^{-4}	-8
Mercuric acetate (dibasic)	1×10^{-4}	-100
CuSO ₄	1×10^{-4}	-100
Ethylenediaminetetra-acetate	1×10^{-2}	0
NaF	1×10^{-2}	-12
NaCN	1×10^{-2}	-22
	5×10^{-2}	-36
(NH ₄) ₂ SO ₄	1×10^{-2}	+57

Among the compounds which are known to affect the metabolism of L-ascorbic acid *in vivo*, only barbiturate (5,5-diethylbarbituric acid) showed a remarkable inhibition for the reduction process, but others, chloretone, antipyrine, 4-aminoantipyrine, and lycorine, had no noticeable effect at the concentration of 3.3×10^{-3} M. The inhibition by barbiturate was also complete in oxidation process at the same concentration. The inhibition caused by barbiturate was shown to be non-competitive to TPN or TPNH by Lineweaver and Burk's analysis (76).

DISCUSSION

From the experiments described above, it can be pointed out that the specificity of TPN L-hexonate dehydrogenase from rat liver depends on the directions in which the reaction is carried out; narrow in the direction of dehydrogenation and broad in that of reduction. Hers (4, 20) described an enzyme named aldose reductase which has a rather broad specificity over various sugar derivatives. The pattern of specificity of the present enzyme for the reduction process closely resembles that of the aldose reductase except for its inactivity on higher sugars. Similar behavior was also observed in their response to sulfate ions and ethylenediaminetetracetate. The insensitivity to some higher aldose was pointed out for the liver enzyme (4, 20), and Hers differentiated this enzyme

hibited the activity even at the concentrations of 10^{-4} M or more. High concentrations of ethylenediaminetetraacetate and sodium azide did not have any appreciable effect on the activity. At pH 7.0, 0.05 M potassium cyanide inhibited the enzyme activity considerably and the inhibition was complete in the oxidation reaction, but it is not clear whether the latter inhibition was caused by the formation of cyanide-TPN complex.

from aldose reductase naming the former aldehyde reductase. The TPN L-hexonate dehydrogenase is considered to have the most close resemblance to the aldehyde reductase except for its inactivity toward ketoses, although the description of Hers is rather incomplete and not definitive. The name of TPN L-hexonate dehydrogenase is thought to be more adequate than that of aldehyde reductase, since the former characterizes the specific aspect in dehydrogenation.

The present experiment demonstrates the direct conversion of D-glucuronolactone to L-gulonolactone, and the opinion presented by Ul Hassan and Lehninger (1) has been disclaimed while the speculation proposed by Hers (20) has been substantiated here. Ishikawa and Noguchi (3) dealt with the enzyme preliminarily, using guinea pig liver and they showed a production of D-glucuronate from L-gulonate through its reverse reaction. Ishikawa (81) also showed that this enzyme can be separated from DPN L-gulonic dehydrogenase, and he concluded that these two enzymes are different from each other. Recently Ashwell *et al.* (7), Kilgore and Starr (6) and Payne and McRorie (5) demonstrated the presence of an enzyme in some bacteria which catalyzes the reversible reduction of some keturonates by DPNH or TPNH. Taking account of the inactivity of the present enzyme on these keturonic acids and DPNH, the hexuronic reductases and this enzyme should be differentiated from each other. Some years ago, Ashwell (24) reported an enzyme from rat liver which catalyzed the conversion of D-galacturonate to L-xylulose with TPN *via* the probable intermediate, L-galactonate and 3-keto-L-gulonate. Although the activities of the present enzyme based in the reduction of D-glucuronolactone and in the oxidation of L-galactonate were in constant relation to each other in the course of purification, neither the formation of L-xylulose nor the evolution of carbon dioxide from L-galactonate by TPN was detected. Therefore, it is doubtful that there exists another enzyme which has the activity as reported by

Ashwell.

It is clear from the data described above that the enzyme is quite different from glyoxylic reductase (82-84), ribulose dehydrogenase which acts on the reduction of D-arabinosone to D-ribulose (85), aldose dehydrogenase derived from *Pseudomonas* (86), D-xylose reductase of *Penicillium chrysogenum* (87), and the enzyme reported by Den *et al.* (88) which catalyzes the reversible reduction of malonic semialdehyde to β -hydroxypropionate.

As shown in the present experiment, the enzyme catalyzes the reduction of a number of sugar derivatives and others, in contrast to the narrow specificity in the oxidation process. Such broad specificity in the reduction process is considered to be due to the action of a single enzyme, for there is a good agreement between the specificities of purified preparation and of crude enzyme. This contrast in specificity depending on the direction of the reaction, namely, broadness in reduction and narrowness in oxidation, signifies a limited reversibility of this enzyme. Such an unilateral pattern of reversibility seems to belong to a rather unparalleled case.

The experimental results except for alduronate as substrate showed that the attacking point of the enzyme is a free aldehyde group and not a keto group. The reactivity of some ketomonosaccharides to aldehyde reductase observed by Hers (4) might be due to an intervention of other enzymes since in his system rather crude preparation was used. Reasoning from the above general rules, erythrose and threose may be presumed to exist as an oxo-form in fairly large amount in aqueous solution, although they were believed to have an oxide ring (89). It has recently been reported that D-glucuronolactone in aqueous solution exists nearly completely in oxo-form (90). However, the data on other related compounds are not available at present. Since it is rather difficult to consider, from the experiment illustrated in Fig. 2, that D-glucuronate becomes accessible through the transformation to its lactone, the reactivity of D-glucuronate or possibly other uronates must be regarded as one exception. The reason

why alduronates and osones from pentose are not accessible remains yet to be elucidated.

The respective formation of L-gulonolactone and L-galactonolactone by the reduction of D-glucuronic and D-galacturonic esters suggests another possibility for physiological formation of these lactones, as has been proposed by M a p s o n (77). In these reactions, the destiny of the separated alcoholic residue is not clear. The reason why the activity of DL-glyceraldehyde is larger than the mean value of the activities of both D- and L-glyceraldehyde is also not clear. This finding is in contrast with the exclusive phosphorylation of the L- form of glyceraldehyde by triokinase (91).

The identification of the product of enzymic reduction of D-glucuronolactone as L-gulonolactone may be hindered by formation of the latter from L-gulonate by lyophilization, which was carried out for the preparation of the chromatographic sample. This possibility was pointed out in the formation of D-glucono- δ -lactone (53). However, the formation of L-gulonolactone was also ascertained by coupling the reaction by this enzyme with that by L-gulonolactone dehydrogenase, while its formation was not detected when D-glucuronate was used as substrate.

The specificity of the enzyme in oxidation is fairly strict with L-hexonate, from which the proposed name of the enzyme originated, and the similar strict configurational specificity has been shown in other enzymes which metabolize aldonates (16, 68, 92, 93).

Both the highest activity of L-iduronate among all uronates tested in reduction and on the other hand, the highest activity of L-idonate among all effective L-hexonates in oxidation may be worth noting because L-iduronate is known to be a constituent of chondroitin sulfate B (94-98), and there may be a possibility that L-iduronate is converted to L-idonate by this enzyme, L-xylulose being produced from the latter by the action of DPN L-gulonate dehydrogenase (92). The enzyme must play an important role in carbohydrate metabolism in various pathways

by virtue of its broad specificity.

Now that the direct conversion of D-glucuronolactone to L-gulonolactone was proved unequivocally, it follows that a new possibility opens for the formation of L-gulonolactone if D-glucuronolactone is really formed in tissue. The argument concerning the possible way under physiological conditons for the formation of L-gulonolactone will be presented elsewhere (99), but the direct conversion stated here might be of little significance because the formation of L-gulonolactone from D-glucuronate has never been proved except the lactonization catalyzed by lactonase I, and this enzyme has lower activity on lactonization with D-glucuronate than with L-gulonate (23). Recently, Payne and McRorie (5) and Kilgore and Starr (6) showed the possibility of intermediary formation of D-fructuronate in the transformation of D-glucuronate to L-gulonate just as the corresponding formation¹ of D-tagaturonate between D-galacturonate and L-galactonate. We also searched for this possibility with crude homogenate and the purified system derived from rat liver, and in both cases it was observed that D-fructuronate and D-tagaturonate were almost completely inactive as substrate while the formation of L-gulonate from D-glucuronate and of L-galactonate from D-galacturonate was observed. This shows the direct reduction of D-glucuronate to L-gulonate and of D-galacturonate to L-galactonate, a result consistent with that of the more recent paper of Kilgore *et al.* (8).

As shown in Table VIII, the enzyme is widely distributed in the organs of various species regardless of the pattern of L-ascorbic acid synthesizing activity (100, 101). Dense presence of the enzyme in the kidney and other findings on its distribution are in accordance with that of Grollman and Lehninger (100) except for the complete inactivity in the spleen of rats. However, D-glucosone and DL-glyceraldehyde were slightly reactive in all the organs tested, and the mechanism are not clear at present.

Among the compounds known to be related to the metabolism of ascorbic acid or glu-

cronic acid, barbiturate is the only agent to inhibit this enzyme reaction. A paradoxical relation between this inhibitory effect and the stimulatory effect on ascorbic acid synthesis upon administration *in vivo* of the drug (102-104) remains to be elucidated. Other known drugs, such as chloretone (102-104), lycorine (105) and antipyrine (106, 107), might affect other points of the reaction sequence of these metabolites.

SUMMARY

1. The enzyme which catalyzes the conversion of D-glucuronate to L-gulonate by TPNH was purified about 500-fold from rat liver. This preparation did not exhibit the activity of lactonase or esterase.

2. Evidence was presented for the direct conversion of D-glucuronolactone to L-gulonolactone by the same enzyme.

3. Stoichiometrical relation was found in the conversion of D-glucuronolactone or ethyl D-glucuronate to L-gulonolactone.

4. Equilibrium constants and other kinetic constants were estimated.

5. Substrate specificity in the reduction reaction was broad with compounds having a free aldehyde group except for uronate, while in the oxidation process the enzyme acted exclusively upon L-hexonate and its lactone. From these findings, the name of TPN L-hexonate dehydrogenase was proposed for the enzyme.

6. The enzyme was widely distributed with almost the same pattern of substrate specificity.

7. The enzyme was inhibited specifically by barbitol.

8. Substrate specificity and the role of the enzyme in the metabolism of L-ascorbic acid were discussed.

The authors are indebted to the members of this laboratory and the persons and companies listed in the text for their kind supply of some of the materials and valuable advices. This work was supported in part by a grant for scientific research from the Ministry of Education.

REFERENCES

- (1) ul Hassan, M., and Lehninger, A. L., *J. Biol. Chem.*, **223**, 123 (1956)
- (2) Hers, H. G., *Biochim. et Biophys. Acta*, **22**, 202 (1956)
- (3) Ishikawa, S., and Noguchi, K., *J. Biochem.*, **44**, 465 (1957)
- (4) Hers, H. G., *Biochim. et Biophys. Acta*, **37**, 120 (1960)
- (5) Payne, W. J., and McRorie, R. A., *Biochim. et Biophys. Acta*, **29**, 466 (1958)
- (6) Kilgore, W. W., and Starr, M. P., *Biochim. et Biophys. Acta*, **29**, 659 (1958)
- (7) Ashwell, G., Wahba, A. J., and Hickman, J., *Biochim et Biophys. Acta*, **30**, 186 (1958)
- (8) Kilgore, W. W., and Starr, M. P., *J. Biol. Chem.*, **234**, 2227 (1959)
- (9) Burns, J. J., Peyser, P., and Moltz, A., *Science*, **124**, 1148 (1956)
- (10) Burns, J. J., and Evance, C., *J. Biol. Chem.*, **223**, 897 (1956)
- (11) Burns, J. J., *Nature*, **180**, 553 (1957)
- (12) Chang, Y. T., and Tung, L., *Acta Physiol. Sinica*, **21**, 190 (1957)
- (13) Chatterjee, I. B., Ghosh, N. C., Ghosh, J. J., and Guha, B. C., *Science*, **126**, 608 (1957)
- (14) Chatterjee, I. B., Chosh, J. J., Ghosh, N. C., and Guha, B. C., *Biochem. J.*, **70**, 509 (1958)
- (15) Yamada, K., Ishikawa, S., and Shimazono, N., *Biochim. et Biophys. Acta*, **32**, 253 (1959)
- (16) Kanfer, J., Burns, J. J., and Ashwell, G., *Biochim. et Biophys. Acta*, **31**, 556 (1959)
- (17) Bublitz, C., and Lehninger, A. L., *Biochim. et Biophys. Acta*, **32**, 290 (1959)
- (18) Chatterjee, I. B., Chatterjee, G. C., Ghosh, N. C., Ghosh, J. J., and Guha, B. C., *Biochem. J.*, **74**, 193 (1960)
- (19) Suzuki, K., Mano, Y., and Shimazono, N., *J. Biochem.*, **47**, 846 (1960)
- (20) Hers, H. G., "Le Métabolisme du Fructose", Bruxelles, p. 133 (1957)
- (21) Yamada, K., *J. Biochem.*, **46**, 361 (1959)
- (22) Winkelman, J., and Lehninger, A. L., *J. Biol. Chem.*, **233**, 794 (1958)
- (23) Yamada, K., *J. Biochem.*, **46**, 529 (1959)
- (24) Ashwell, G., *Federation Proc.*, **16**, 146 (1957)
- (25) Mano, Y., Yamada, K., Suzuki, K., and Shimazono, N., *Biochim. et Biophys. Acta*, **34**, 563 (1959)
- (26) Dixon, M., and Webb, E. C., "Enzymes", Longmans, Green and Co., London, p. 201 (1958)
- (27) Mano, Y., Suzuki, K., Yamada, K., and Shima-

- zono, N., *Biochem. Biophys. Res. Comm.*, **3**, 136 (1960)
- (28) Jansen, E. F., and Jang, R., *J. Am. Chem. Soc.*, **68**, 1475 (1946)
- (29) Ishidate, M., and Takitani, S., *Chem. Pharm. Bull.*, **7**, 291 (1958)
- (30) Shafizadeh, F., and Wolfrom, M. L., *J. Am. Chem. Soc.*, **77**, 2568 (1955)
- (31) Machida, S., *J. Chem. Soc. Japan*, **70**, 82 (1949)
- (32) Ameyama, M., and Kondo, K., *Bull. Agr. Chem. Soc. Japan*, **22**, 380 (1958)
- (33) Lucas, H. J., and Baumgarten, W., *J. Am. Chem. Soc.*, **63**, 1653 (1941)
- (34) Wislicenus, W., Böklen, E., and Reuthe, F., *Ann. Chem.*, **363**, 340, 353 (1908)
- (35) Harries, C., and Tank, L., *Ber. deut. chem. Ges.*, **41**, 1701 (1908)
- (36) Wislicenus, W., *Ber. deut. chem. Ges.*, **20**, 2930 (1887)
- (37) Neuberg, C., *Biochem. Z.*, **24**, 166 (1910)
- (38) Becker, C. E., and Day, H. G., *J. Biol. Chem.*, **201**, 795 (1955)
- (39) Reley, H. L., Morley, J. F., and Friend, N. A. C., *J. Chem. Soc.*, 1875 (1932)
- (40) Wohl, A., and Lange, M., *Ber. deut. chem. Ges.*, **41**, 3612 (1908)
- (41) Nef, J. U., *Ann. Chem.*, **335**, 191 (1904)
- (42) Claisen, L., *Ber. deut. chem. Ges.*, **36**, 3664 (1903)
- (43) Harries, C., *Ber. deut. chem. Ges.*, **34**, 1488 (1901)
- (44) Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **128**, 465 (1939)
- (45) Perlin, A. S., and Brice, C., *Canad. J. Chem.*, **33**, 1216 (1955)
- (46) Hockett, R. C., *J. Am. Chem. Soc.*, **57**, 2260 (1935)
- (47) Schmidt, T., and Treiber, R., *Ber. deut. chem. Ges.*, **66**, 1765 (1933)
- (48) Wolfrom, M. L., *J. Am. Chem. Soc.*, **51**, 2188 (1929)
- (49) Fischer, E., and Hess, K., *Ber. deut. chem. Ges.*, **45**, 912 (1912)
- (50) Schaffer, R., and Isbell, H. S., *J. Res. Nat. Bur. Stand.*, **56**, 191 (1956)
- (51) Wolfrom, M. L., and Anno, K., *J. Am. Chem. Soc.*, **74**, 5583 (1952)
- (52) Mapson, L. W., and Breslow, E., *Biochem. J.*, **68**, 395 (1958)
- (53) Isbell, H. S., Karabinos, J. V., Frush, H. L., Holt N. B., Schwebel, A., and Galkowski, T. T., *J. Res. Nat. Bur. Stand.*, **48**, 163 (1952)
- (54) Hudson, C. S., and Isbell, H. S., *J. Am. Chem. Soc.*, **51**, 2225 (1929)
- (55) Phelps, F. P., and Bates, F. J., *J. Am. Chem. Soc.*, **56**, 1250 (1934)
- (56) Fischer, E., and Fay, I. W., *Ber. deut. chem. Ges.*, **28**, 1975 (1895)
- (57) Glatthaar, C., and Reichstein, T., *Helv. Chim. Acta*, **21**, 3 (1938)
- (58) Fischer, E., and Herborn, H., *Ber. deut. chem. Ges.*, **29**, 1961 (1896)
- (59) Hudson, C. S., *J. Am. Chem. Soc.*, **73**, 4498 (1951)
- (60) Clowes, G. H. A., and Tollens, B., *Ann. Chem.*, **310**, 166 (1899)
- (61) Hardegger, E., Kreis, K., and El Khadem, H., *Helv. Chim. Acta*, **34**, 2343 (1951)
- (62) Wohl, A., and Schellenberg, R., *Ber. deut. chem. Ges.*, **55**, 1404 (1922)
- (63) Wislicenus, J., *Ann. Chem.*, **166**, 3 (1873)
- (64) Levene, P. A., and Walti, A., "Organic Syntheses," Coll. Vol. **II**, John Wiley and Sons Inc., New York, p. 5 (1946)
- (65) Beisenherz, C., Bücher, T., and Garbade, K.H., "Methods in Enzymology", Vol. **I**, Academic Press Inc., New York, p. 391 (1955)
- (66) Willstätter, R., and Schudel, G., *Ber. deut. chem. Ges.*, **51**, 780 (1918)
- (67) Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 751 (1956)
- (68) Suzuki, K., Mano, Y., and Shimazono, N., *J. Biochem.*, **48**, 313 (1960)
- (69) Ochoa, S., "Methods in Enzymology", Vol. **I**, Academic Press Inc., New York, p. 699 (1955)
- (70) Shibata, K., Benson, A. A., and Calvin, M., *Biochim. et Biophys. Acta*, **15**, 461 (1954)
- (71) Ishidate, M., and Nambara, T., *Chem. Pharm. Bull.*, **6**, 515 (1957)
- (72) Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183**, 123 (1950)
- (73) Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, **147**, 399 (1943)
- (74) Eisenberg, F., and Field, J. B., *J. Biol. Chem.*, **222**, 293 (1956)
- (75) Metzenberg, R. L., and Mitchell, H. K., *J. Am. Chem. Soc.*, **76**, 4187 (1954)
- (76) Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934)
- (77) Mapson, L. W., and Isherwood, F. A., *Biochem. J.*, **64**, 13 (1956)
- (78) Roe, J. H., Epstein, J. H., and Goldstein, N. P., *J. Biol. Chem.*, **178**, 839 (1949)
- (79) Baudisch, O., *Biochem. Z.*, **89**, 279 (1918)
- (80) Watanabe, M., and Arai, Y., *Symposia on Enzyme Chemistry*, **9**, 201 (1956)
- (81) Ishikawa, S., *J. Biochem.*, **46**, 347 (1959)
- (82) Zelitch, I., *J. Biol. Chem.*, **201**, 719 (1953)
- (83) Zelitch, I., *J. Biol. Chem.*, **216**, 553 (1955)
- (84) Katagiri, H., Tochikura, T., and Imai, K.,

- Symposia on Enzyme Chemistry* **12**, 125 (1960)
- (85) Uehara, K., Sugano, K., Mizoguchi, T., and Takeda, S., *Vitamins*, **19**, 165 (1960)
- (86) Katagiri, M., Maeno, H., and Hori, K., *J. Biochem.*, **45**, 825 (1958)
- (87) Chang, C., and Knight, S. G., *Biochim. et Biophys. Acta*, **35**, 454 (1959)
- (88) Den, H., Robinson, W. G., and Coon, M. J., *J. Biol. Chem.*, **234**, 1666 (1959)
- (89) Freudenberg, W., *Ber. deut. chem. Ges.*, **65**, 168 (1932)
- (90) Kawada, M., unpublished.
- (91) Bubnitz, C., and Kennedy, E. P., *J. Biol. Chem.*, **211**, 951 (1954)
- (92) Machiyama, Y., Mano, Y., and Shimazono, N., *Biochim. et Biophys. Acta*, **42**, 564 (1960)
- (93) Smiley, J. D., and Ashwell, G., *Federation Proc.*, **19**, 85 (1960)
- (94) Hoffman, P., Linker, A., and Meyer, K., *Science*, **124**, 1252 (1956)
- (95) Cifonelli, J. A., Ludowieg, J., and Dorfman, A., *J. Biol. Chem.*, **233**, 541 (1958)
- (96) Satake, M., and Masamune, H., *Tohoku J. Exptl. Med.*, **68**, 44 (1958)
- (97) Satake, M., *Tohoku J. Exptl. Med.*, **68**, 385 (1958)
- (98) Stoffyn, P. J., and Jeanloz, R. W., *J. Biol. Chem.*, **235**, 2507 (1960)
- (99) Shimazono, N., and Mano, Y., *Ann. New York Acad. Sci.*, **92**, Art. 1, 91 (1961)
- (100) Grollman, A. P., and Lehninger, A. L., *Arch. Biochem. Biophys.*, **69**, 458 (1957)
- (101) Roy, R. N., and Guha, B. C., *Nature*, **182**, 319 (1958)
- (102) Burns, J. J., Mosbach, E. H., and Schulenberg, S., *J. Biol. Chem.*, **207**, 679 (1954)
- (103) Burns, J. J., Evance, C., and Trousof, N., *J. Biol. Chem.*, **227**, 785 (1957)
- (104) Burns, J. J., *J. Am. Chem. Soc.*, **79**, 1257 (1957)
- (105) Mineshita, T., Yamaguchi, K., and Yamamoto, K., *Proc. Japan Acad.*, **35**, 405 (1959)
- (106) Longenecker, H. E., Fricke, H. H., and King, C. G., *J. Biol. Chem.*, **135**, 497 (1940)
- (107) Conney, A. H., Gastel, R., and Burns, J. J., *Federation Proc.*, **18**, 378 (1959)

Toxicity of Cyclic Compound of β -Eleostearic Acid*

By NOBORU MATSUO

(From the Department of Chemistry, Seikei University, Musashino-shi, Tokyo)

(Received for publication, February 24, 1961)

When rats were fed on diet containing about 20 per cent of thermally polymerized oil, intoxication of the rats was observed, and the symptom was particularly acute in case such a highly unsaturated oil as fish oil was used as raw material (1-3).

The cause of toxicity was ascertained to be the cyclic monomer formed by the thermal polymerization previously reported (4, 5). This report concerns the preparation of a cyclic compound of β -eleostearic acid and with its toxicity for rats.

EXPERIMENTALS AND RESULTS

Preparation of Pure β -Eleostearic Acid—Tung oil, indicated in Table I-i, was isomerized by the method of Hoffman *et al.* (6). About 500 g. of tung oil were mixed with 1 ml. of saturated potassium iodide solution, and the

mixture was thoroughly stirred, kept air-tight and exposed to the sun-light to accelerate isomerization. Then the oil was saponified with alkali as usual and fatty acid was obtained. This was washed with distilled water, dissolved in ethanol and let stand overnight at -15°C . The precipitated β -eleostearic acid was filtered and dried in vacuum. The melting point of the acid was $70-71^{\circ}\text{C}$, and its infrared absorption spectrum was as shown in Fig. 1-a, exactly the same as that reported by Hoffman *et al.* (6).

Then ethyl ester of β -eleostearic acid was prepared as usual and distilled in vacuum. The fraction collected at $193-196^{\circ}\text{C}$ showed the properties indicated in Table I-ii, which were almost comparable to the theoretical values. The infrared absorption spectrum of this ester was shown in Fig. 1-b.

TABLE I
Properties of Samples

No.	Sample	n_D^{20}	Iodine Value (Wijs)	Saponification Value	Acid Value	Molecular Weight (Rast)
i	Tung oil	1.5063	173.7	194.2	5.4	—
ii	β -Eleostearic acid ethyl ester	1.4892	165.5 (166.0)	182.2 (183.0)	2.0	301 (306)
iii	β -Eleostearic acid ethyl ester-acrolein adduct compound	1.4847	139.8 (140.3)	153.6 (154.7)	1.6	367 (362)

() shows theoretical value.

* A brief account of this paper was reported at the annual meeting of the Japan Chemical Society held in Tokyo in April, 1958. The full text written in Japanese appeared in *Nippon Kagaku Zasshi*, **81**, 467 (1960).

Preparation of Adduct Compound of β -Eleostearic Acid Ethyl Ester with Acrolein—The preparation was carried out according to the method of Miyoshi and Kurata (7) as shown in Table II.

TABLE II

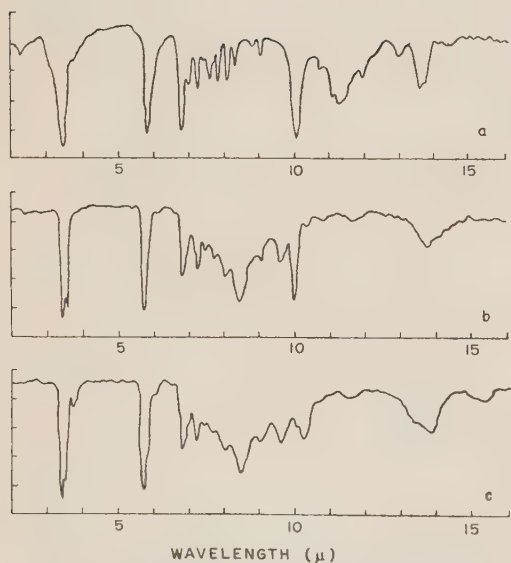
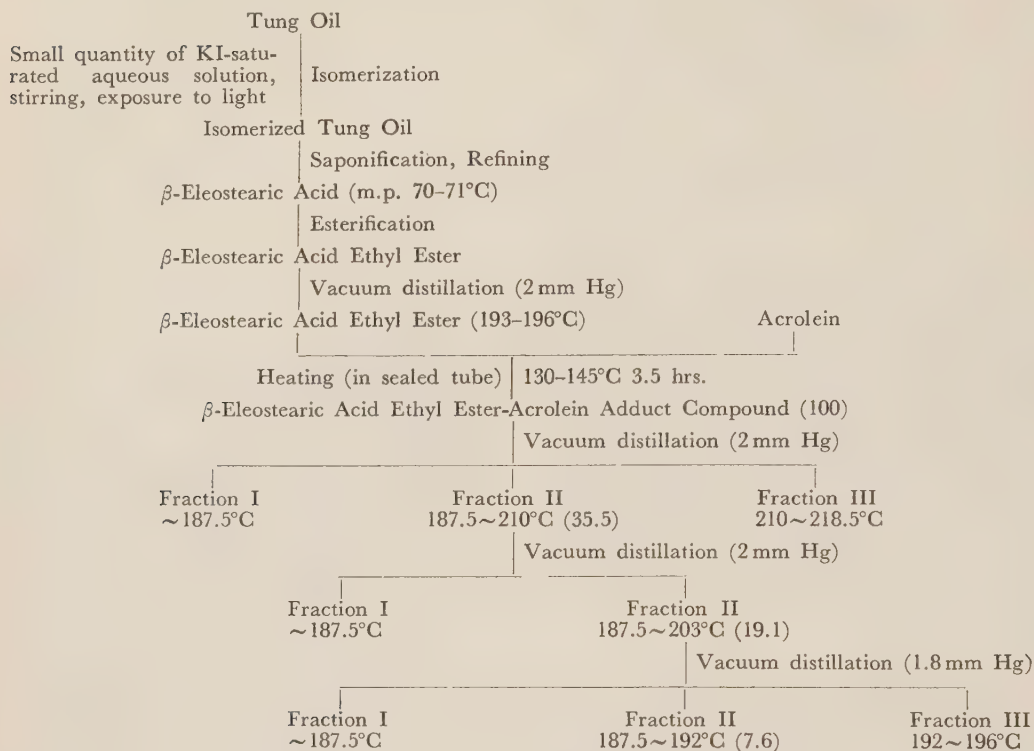
Preparation of β -Eleostearic Acid Ethyl Ester-Acrolein Adduct Compound

FIG. 1. Infrared absorption spectrum.

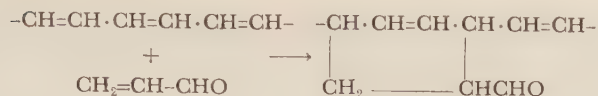
a. β -eleostearic acid, b. β -eleostearic acid ethyl ester, c. β -eleostearic acid ethyl ester-acrolein adduct compound.

The β -eleostearic acid ethyl ester and acrolein were mixed in sealed tube in the ratio of 4:1 by weight, and heated at 130–145°C for 3.5 hours. The reaction product was distilled in vacuum. The distillation was repeated three times and the final distillate at 187.5–192°C/1.8 mm Hg was collected (yield 7.6 per cent).

This fraction was transparent, light yellowish, and contained no acrolein when tested by the reaction with sodium nitroprusside and piperidine (8). The properties of adduct compound was shown in Table I-iii, comparable to the theoretical value. Its infrared absorption spectrum was also shown in Fig. 1-c.

β -Eleostearic acid ethyl ester has three conjugated double bonds, whose configurations are all trans form. Cyclohexene ring as shown in the following equation might be formed by the addition of acrolein though Diels-Alder reaction.

When the infrared absorption spectrum



of β -eleostearic acid ethyl ester-acrolein adduct compound was compared with that of the original β -eleostearic acid ethyl ester, the following difference was observed:

The strong absorption of $10.1\ \mu$ (990 cm^{-1}) due to the existence of conjugated trans-trans double bond, which was found in β -eleostearic acid ethyl ester, almost disappeared as a result of the addition of acrolein, and $10.3\ \mu$ (970 cm^{-1}) absorption, due to trans double bond appeared on the other hand. $15.2\ \mu$ (660 cm^{-1}) absorption was not found in the spectrum of the original ester. This absorption band was also ascertained by MacDonald (9) in the monomeric, non-urea-adduct-forming material from the ethanolysis of heated linseed oil. Cyclohexene has a band 670 cm^{-1} ($14.97\ \mu$) (9).

Bands at 663 and 671 cm^{-1} (15.1 and $14.9\ \mu$) are characteristic of Δ^2 - and Δ^3 -steroids, respectively (10). Cis α -ionone (11) also absorbs in this region. This absorption may be attributable to the cyclohexene ring. Another absorption, due to the aldehyde radical, was observed at $3.7\ \mu$ (2700 cm^{-1}).

These differences seem to be caused by the fact that the addition of acrolein to β -eleostearic acid ethyl ester as pictured in the above formula.

From the properties and infrared absorption spectrum, the product obtained might be the adduct compound of one molecule of acrolein with one molecule of β -eleostearic acid ethyl ester.

Preparation of Intra-Molecular Cyclic Compound

TABLE III

Separation of Cyclic Monomer from Thermally Treated Product of β -Eleostearic Acid Ethyl Ester

β -Eleostearic Acid Ethyl Ester (100)	
Heating (in sealed tube) $180\text{--}185^\circ\text{C}$, 5 hrs.	
Thermally Treated Product from β -Eleostearic Acid Ethyl Ester (99)	
Vacuum distillation (3 mm Hg)	
Loss (4.8)	Distillate $161.5\text{--}206^\circ\text{C}$ (75.5)
Residue (18.7)	
Urea-adduct forming method Stand overnight (2.5°C)	
Urea (5 volumes) Ethanol (8 volumes) $50\text{--}55^\circ\text{C}$	
Filtrate I (Not forming urea-adduct)	Urea-Forming Adduct
Concentration, Stand overnight (2.5°C)	
Crystal of Urea Separated	Washing with ethanol Hot water
Filtrate II	Straight Chain Ethyl Ester (60.1)
Concentration	
Stand 48 hrs. ($3\text{--}4^\circ\text{C}$)	
Crystal of Urea Separated	Filtrate III
Ethanol removed	
Reddish Brown Ester (8.4)	
Vacuum distillation (2 mm Hg)	
Fraction I $109\text{--}168^\circ\text{C}$ (2.0)	Fraction II $168\text{--}174.5^\circ\text{C}$ (4.0)
Transparent, light yellow	
Residue (1.1)	
Loss (1.3)	

of β -Eleostearic Acid Ethyl Ester—The process of preparation was shown in Table III. After heating 230 g. of β -eleostearic acid ethyl ester in a sealed tube at 180–185°C for 5 hours, 228 g. of the contents of the tube were distilled in vacum. The distillate at 161.5–206°C/3 mm Hg was collected. The yield was 174 g. (75.5 per cent).

The distillate was dissolved in 1.5 liters of ethanol and was gradually added with 870 g. of urea at 50–55°C. After thoroughly stirring for 30 minutes, the mixture was let stand overnight. The precipitation of urea-adduct was collected and washed with 500 ml. of ethanol (washings were added to the filtrate).

By treating the precipitate with a large quantity of hot water, straight chain ester was separated from the precipitate. Its yield was 60.1 per cent for the original β -eleostearic acid ethyl ester.

The mixture of filtrate and washings was concentrated to about 800 ml. and kept overnight at 2.5°C. The crystal of urea was removed and the filtrate II was again concentrated to 500 ml. and kept at 3–4°C for 48 hours. In this way, filtrate III was obtained free from any precipitates. When ethanol was evaporated out of this filtrate, a residue of reddish brown colour was obtained. Its yield was 8.4 per cent.

Then the residue was distilled in vacuum (2 mm Hg) and the distillate at 168–174.5°C was collected. The yield of the fraction was 4.0 per cent.

It was transparent, light yellow liquid with properties as shown in Table IV, and its main component might be a cyclic mono-

mer of β -eleostearic acid ethyl ester. Its infrared absorption spectrum was shown in Fig. 2.

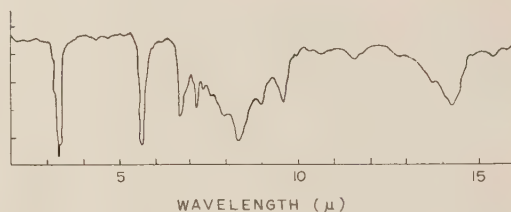


FIG. 2. Infrared absorption spectrum of cyclic monomer of β -eleostearic acid ethyl ester.

When the spectrum in Fig. 2 was compared with the spectrum of the original β -eleostearic acid ethyl ester, the following difference was observed:

The strong 10.1 μ (990 cm^{-1}) absorption in the spectrum of β -eleostearic acid ethyl ester, which was due to the conjugated trans-trans double bond, almost disappeared, and new absorption due to cis double bond appeared at 14.4 μ (690 cm^{-1}). This seems to be caused by the formation of double bond in a cis ring, replacing conjugated trans-trans double bond, as a result of cyclization of β -eleostearic acid ethyl ester caused by heating. New weak absorption at 15.2 μ seems to be caused by the cyclohexene ring.

From the above mentioned findings on molecular weight, infrared absorption spectrum and non adduct forming character with urea, it is known that the main component of the distillate at 168–174.5°C is a cyclic monomer produced by intra-molecular cyclization of β -eleostearic acid ethyl ester.

Toxicity of Cyclic Compound—When rats, each weighing 60 to 70 g., were fed on basal

TABLE IV

Properties of Cyclic Monomer Separated from Thermally Treated Product of β -Eleostearic Acid Ethyl Ester

Sample	n_D^{20}	Iodine Value (Wijs)	Saponification Value	Acid Value	Molecular Weight (Rast)
Cyclic Monomer of β -Eleostearic Acid Ethyl Ester	1.4781	115.3	164.4	4.0	300 (306)

() shows theoretical value.

diet (Table V), containing 10 per cent of the β -eleostearic acid ethyl ester, the rats grew normally.

TABLE V
Composition of Basal Diet (%)

Starch (Rice Powder)	75
Casein (Ether Extracted)	9
McCullum Salt Mixture	3
Yeast	3
Liver Oil	One drop a day
Sample	10

(i) *Toxicity of Eleostearic Acid Ethyl Ester-Acrolein Adduct Compound for Rats*—Rats, each weighing about 60 g., were fed on basal diet (Table V), containing 10 per cent of the adduct compound of β -eleostearic acid ethyl ester with acrolein.

In this experiment, as shown in the growth curves given in Fig. 3, all rats died

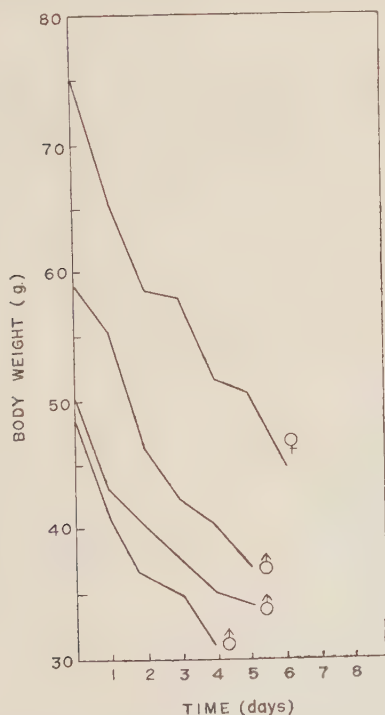


FIG. 3. Growth curves of rats fed on basal diet containing 10 per cent of adduct compound of β -eleostearic acid ethyl ester with acrolein (all rats died at the end of the curves).

in 4 to 6 days after showing an abrupt decrease in body weight.

(ii) *Toxicity of Cyclic Monomer Obtained from Thermally Treated Product of Eleostearic Acid Ethyl Ester*—Rats, weighing about 60 g. each, were fed on basal diet containing 10 per cent of cyclic monomer separated from thermally treated product of β -eleostearic acid ethyl ester as mentioned above. In this experiment, as illustrated in the growth curves given in Fig. 4, all rats died in 7 days or less after showing a remarkable loss of weight.

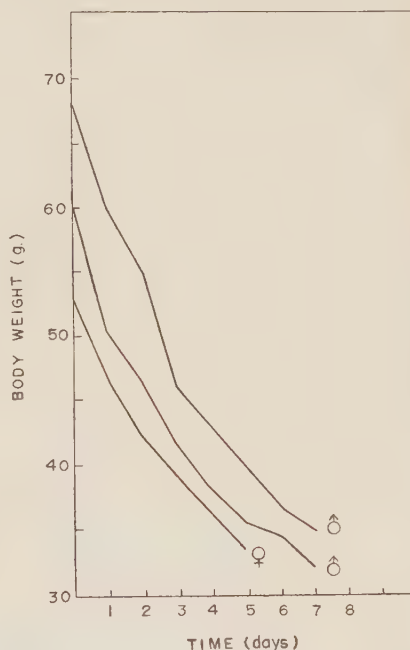


FIG. 4. Growth curves of rats fed on basal diet containing 10 per cent of cyclic monomer separated from heated β -eleostearic acid ethyl ester (all rats died at the end of the curves)

DISCUSSION

Thermally polymerized oil, obtained by heating cuttlefish oil in a carbon dioxide stream or in the air at 250°C for 10 hours (no catalyser), has toxicity even though it contains an extremely small quantity of peroxide. When rats, each weighing 50 to 70 g., were fed on basal diet containing 20 per cent of this thermally polymerized oil, they all died within 15 days. As a result of ethanoly-

sis of the thermally polymerized oil and subsequent separation into straight chain and cyclic compounds by the urea adduct, it was found that the toxicity was almost attributable to the cyclic constituent (1, 2).

When refined rape-seed oil was used as a sample, it was also observed that cyclic product was responsible for toxicity in the case of heat treatment of the oil at 250°C for 50 hours (3). Furthermore, when cyclic ethyl ester, separated from the thermal polymer of highly unsaturated fatty acid ethyl ester and ethyl linolenate (4, 5) was distilled into distillable ester and non-distillable ester, it was found that the distillable ester shows particularly acute toxicity and that the non-distillable ester has only weak toxic effect. Judging from the boiling point of the distillable ester obtained when ethyl linolenate was used as a sample, and also comparing the molecular weight and other properties of the distillable fraction with those of the original ester, it was confirmed that the main origin of toxicity was due to the cyclic monomer. According to infrared absorption spectrum, it was also found that this cyclic monomer contained a cyclohexene structure in the molecule.

Several studies (12, 13) were reported concerning the cyclization of β -eleostearic acid because it has three double bonds in a trans form, which was suitable for such study. Therefore, research on cyclization was conducted, using β -eleostearic acid, and toxicity for rats were examined with its cyclic product.

The mixture of β -eleostearic acid ethyl ester and acrolein, even by heating in a sealed tube, formed a cyclohexene ring by the addition reaction. Thermally treated product was thrice distilled in vacuum, and distillate at 187.5–192°C/1.8 mm Hg. was obtained. Judging from its molecular weight and infrared absorption spectrum, the main component of this fraction has probably a cyclic structure.

Cyclic monomer was obtained by heating β -eleostearic acid ethyl ester under such moderate conditions as at 180–185°C for 5 hours, minimizing the formation of dimer or any products other than monomer. Although its yield is small, judging from its molecular

weight and infrared absorption spectrum, it is evidently a monomer containing intra-molecular cyclic constituent.

In animal experiments, in which rats were fed on basal diet containing each one of these products, the rats unexceptionally died in 7 days or less. This fact gave further endorsement to the previous reports that the main origin of toxicity is the cyclic monomer.

SUMMARY

1. An adduct compound of β -eleostearic acid ethyl ester and acrolein having cyclohexene ring in its molecule was obtained. This showed clear toxicity when rats were fed on basal diet containing 10 per cent of it.

2. An intramolecular cyclic compound obtained by heating β -eleostearic acid ethyl ester were found also quite toxic to rats.

3. The cyclic constituents in the above substances were confirmed by infrared absorption spectrum and other several properties.

The author expresses his heartfelt thanks to Dr. K. Kodama, President of Tokushima University, for his continual guidance given in the course of this experiment. Deep appreciation is expressed to Dr. S. Tanaka of Tokyo University's Over-all Research Laboratory for his assistance in the infrared spectrum research. Thanks are also due to Mr. K. Kuwamoto for his cooperation in the experiment.

REFERENCES

- (1) Matsuo, N., *J. Japan. Biochem. Soc.*, **29**, 885 (1958)
- (2) Matsuo, N., *J. Soc. Food and Nutrition (Japan)*, **10**, 255 (1958)
- (3) Matsuo, N., *J. Soc. Food and Nutrition (Japan)*, **12**, 118 (1959)
- (4) Matsuo, N., *J. Japan Oil Chem. Soc.*, **9**, 37 (1960)
- (5) Matsuo, N., *J. Soc. Food and Nutrition (Japan)*, **12** (1959)
- (6) Hoffman, J. S., Oconnor, R. T., Heinzelman, D. C., and Bickford, W. G., *J. Am. Oil Chem. Soc.*, **34**, 338 (1957)
- (7) Miyoshi, S., and Kurata, M., *J. Chem. Soc. (Japan)*, **63**, 1158 (1942)
- (8) Feigl, F., "Spot Tests in Organic Analysis", p. 387 (5th Edition), Elsevier Publishing Co., New York.

- (9) Macdonald, J. A., *J. Am. Oil Chem. Soc.*, **33**, 394 (1956)
- (10) Jones, R. N., and Herling, F., *J. Org. Chem.*, **19**, 1252 (1954)
- (11) Buchi, G., and Yang, N. C., *Helv. Chim. Acta.*, **38**, 1338 (1955)
- (12) Rivett, D. E. A., *J. Am. Oil Chem. Soc.*, **33**, 635 (1956)
- (13) Paschke, R. F., and Wheeler, D. H., *J. Am. Oil Chem. Soc.*, **32**, 473 (1955)

Studies on the Lipogenesis in Animal Tissues under Pathological Conditions

I. The Formation of Unsaturated Fatty Acid in Diabetic and Fasted Rats

By YOH IMAI

(From the Department of Biochemistry, Hokkaido University School of Medicine, Sapporo)

(Received for publication, March 6, 1961)

Since Stetten and Boxer (1) have first shown that the synthesis of fatty acids in the diabetic animal is decreased, many workers reported on the lipogenesis in diabetes as well as in starvation. They were generally agreed in finding out a distinct depression of the fatty acid synthesis by these metabolic lesions (2-4), although the results were grossly divergent regarding cholesterol.

However, references as to the formation of unsaturated fatty acids in these cases, either by synthesis from acetate or by desaturation of corresponding saturated ones, are not as yet available.

According to Weiss *et al.* (5), monounsaturated long chain fatty acid seems indispensable for the synthesis of triglycerides in living organisms, while Andreassen and Stier (6) suggested the need of either linoleic, linolenic or oleic acid for the growth of yeast. On the other hand, Bloomfield and Bloch (7) reported for the first time on a possible mechanism for the desaturation of long chain fatty acid. In these and other respects the unsaturated fatty acids seem to raise questions worthy of further investigations.

The present paper will describe the experiments on the rate of fatty acid synthesis, especially that of unsaturated fatty acid from acetate and the rate of desaturation of saturated fatty acid in diabetic and fasted animal tissues, as a part of the study on lipogenesis under pathological conditions.

EXPERIMENTAL

Animals—Male rats of the Wistar strain, weighing 150 to 200 g. were used throughout. Five mg. of alloxan monohydrate per 100 g. of body weight were

injected intraperitoneally into animals that had been fasted for 24 hours. All rats were maintained on synthetic rat chow of this university* *ad libitum*, which contained approximately 46 per cent of carbohydrate, 5 per cent of fat and 23 per cent of protein. The diabetic animals were sacrificed 10 to 14 days after alloxan injection (mostly once, in two cases twice) when their blood sugar levels were over 160 mg. per 100 ml. of blood. Animals fasted for 24 hours were used as the starved.

Tissue Preparation and Incubation Procedure—In slice experiments, approximately 850 mg. of tissue slices were incubated with 8 ml. of Krebs-Ringer phosphate buffer (8), pH 7.4, which contained 15×10^5 c.p.m. of sodium acetate-1- C^{14} . The medium was aerated with oxygen by shaking for 2 hours at 37°C, and then the lipid was extracted from the slices.

In the experiment with palmitic acid-1- C^{14} , fresh homogenates of rat liver (10 per cent) were prepared in 0.25 M glucose solution at 0°C in a Potter-Elvehjem type all glass homogenizer. Cellular debris and nuclei were removed from the homogenates by centrifugation for 15 minutes at $2,000 \times g$. From the supernatant fluid a mitochondrial fraction was obtained by centrifugation for 10 minutes at $10,000 \times g$, and a microsomal fraction was likewise prepared from the supernatant fraction by centrifuging at $20,000 \times g$ for 40 minutes after removal of the mitochondrial fraction.

In the incorporation experiment, the greater enzymatic activity was found in the microsomal and supernatant fractions than in mitochondrial fraction alone as may be seen in Table III. Thus the procedures were simplified as follows:

The fresh homogenates were immediately centrifuged at $10,000 \times g$ for 10 minutes and the supernatant

* The composition in 10 Kg. of rat chow is as follows (in Kg.): Wheat flour 2.5, wheat bran 1.5, rice-bran 1.5, cornflour 1.5, fishmeal 1.5, defatted soy bean flour 1.5, cod liver oil 0.15, yeast 0.2, and NaCl 0.05.

fluid was removed by suction, leaving the sediment undisturbed. The supernatant fraction was centrifuged again for 10 minutes ($10,000\times g$) for complete removal of mitochondria. Three to five ml. of the whole supernatant fraction were incubated with palmitic acid- 1-C^{14} ($10,000\text{ c.p.m.}$, in Tween 80) in Krebs-Ringer phosphate buffer (θ) pH 7.4, in a total volume of 8 ml. The content of glucose and total nitrogen was estimated in each tissue preparation.

Fatty Acid Extraction—The lipids extracted from the tissue slices with ethanol-ether (2:1) were fractionated into acetone-insoluble and soluble lipids by usual methods. Each lipid fraction was saponified with methanolic KOH, and the unsaponified lipids were removed with ether from the concentrated soap mixture. The remaining mixture was acidified and then fatty acid was extracted with ether. The ether solution was dried on anhydrous sodium sulfate.

When palmitic acid- C^{14} was used as substrate, isolation procedure was simplified as follows: After the addition of 1 ml. of 4*N* hydrochloric acid to the incubation mixture, the flasks were heated at 100°C for 10 minutes. After cooling in a refrigerator, fatty acid was directly extracted from the incubation mixture with three portions of cold ether. The combined ether solution was dried on anhydrous sodium sulfate.

Separation of Saturated and Unsaturated Fatty Acids—Saturated and unsaturated fatty acids were separated according to Bergström and Pääbo (9) with slight modifications (7). The fatty acid mixture (up to 5 mg.) was dissolved in 1.5 ml. of ethylformate. After the addition of 1 ml. of 85% formic acid and 0.2 ml.

of 30% hydrogen peroxide, the mixture was warmed at 40°C for 3 hours in a closed system. The reaction mixture was then evaporated to dryness under reduced pressure, and if necessary, the evaporation was repeated after the addition of toluene. The residue was dissolved in a few ml. of ether and esterified by diazomethane. The solvent was evaporated again.

The methyl esters, weighing 0.5 to 5 mg., were chromatographed on a $0.8\times 20\text{ cm.}$ column containing 1.0 g. of silica gel (100 mesh, obtained from the Kanto Chem Co., and dried at 100°C before use).

The saturated acid esters were eluted from the column by small portions of dichloromethane (12 ml. in total). This fraction was designated as Fraction I.

The subsequent elution of dihydroxy acids esters derived from monounsaturated fatty acids was carried on by dichloromethane containing 1% methanol (three or four successive small portions—12 ml. in total). The fraction was named Fraction II.

Thereafter, the tetrahydroxy acid derivatives originated in diunsaturated acids were eluted by several portions of dichloromethane-methanol (97:3) mixture (12 ml. in total). This fraction was named Fraction III.

In this method, 9:10 monounsaturated acid is oxidized by performic acid to its corresponding 9, 10-dihydroxy acid, while saturated acid remains unchanged. Even when reaction intermediates (10) were formed, the whole 9:10 unsaturated derivatives are eluted from the column together with the dihydroxy acid derivative.

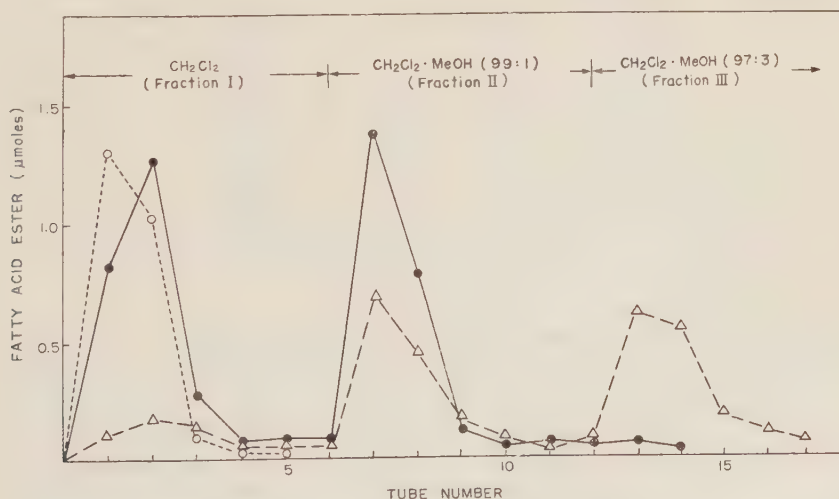


FIG. 1. Elution pattern of authentic fatty acids. Fatty acids (—●— stearic and oleic acids mixture, ...○... palmitic acid and ...△... linoleic acid alone) were applied individually after hydroxylation and esterification.

By the performic oxidation, linoleic acid is not completely converted to tetrahydroxy derivatives (9, 11). About a half portion of the former remains as dihydroxy derivative as is seen in Fig. 1, which may enter the Fraction II.

However, the incorporation of radiocarbon either from acetate- C^{14} or from palmitate- C^{14} into Fraction III which is regarded to contain hydroxy acid derivatives from polyunsaturated fatty acid was negligible as can be imagined from the fact that the synthesis of polyunsaturated fatty acid does not proceed in animal tissues. Consequently, the radioactivity in the dihydroxy acid fraction indicates that it was derived from monounsaturated fatty acid alone. The above method is therefore considered satisfactory for the present purpose.

The amount of the fatty acid ester was determined colorimetrically by making use of the hydroxamic

acid method (12), and the radioactivity of the sample was determined directly on weighed planchets in a gas flow counter (Nuclear-Chicago Co.).

Materials—Palmitic acid- $l\text{-}C^{14}$ and sodium acetate- $l\text{-}C^{14}$ were purchased from the Daiichi Pure Chemicals Co. Triphosphopyridine nucleotide was prepared in this laboratory from sheep liver by the procedure of Le Page and Mueller (13).

RESULTS

Fatty Acid Synthesis from Acetate—As already observed (14), lipogenesis from acetate increased markedly when tissues were incubated in an atmosphere of oxygen. Radiocarbon from acetate- $l\text{-}C^{14}$ was incorporated into fatty acids of different origins in different rates. The results were shown in Table I.

TABLE I

Fatty Acid Synthesis from Acetate- $l\text{-}C^{14}$ in Tissue Slices of Normal Rats

Approximately 850 mg. of tissue slices were incubated in Krebs-Ringer phosphate buffer (pH 7.4) with sodium acetate- $l\text{-}C^{14}$ (15×10^5 c. p. m.) for two hours at 37°C under aerobic conditions.

a) Acetone-Insoluble Lipids

Tissues	Saturated Acids Extracted		Unsaturated Acids Extracted		Rate of Incorporation into Unsaturated Acid $\frac{(b)}{(a)+(b)} \times 100$
	Amount	Total Act. (a)	Amount	Total Act. (b)	
	mg.	c. p. m.	mg.	c. p. m.	%
Liver	1.1	2,220	1.2	2,820	56
Brain	6.3	2,770	7.2	2,590	48
Lung	1.3	3,500	2.7	5,020	59
Heart	0.3	179	0.3	187	51
Kidney	3.4	1,830	3.6	3,450	65
Testis	1.2	1,630	1.8	3,880	70
Intestine	1.4	3,420	1.1	2,970	46

b) Acetone-Soluble Lipids

Liver	1.3	5,800	1.9	8,250	59
Brain	0.4	110	0.4	124	53
Lung	1.3	2,200	1.3	1,375	39
Heart	0.7	1,100	0.9	895	45
Kidney	2.0	1,180	3.2	1,360	54
Testis	2.3	1,010	3.5	1,930	66
Intestine	4.1	15,900	8.4	13,800	47

TABLE II

Fatty Acid Synthesis from Acetate-1-C¹⁴ in Tissue Slices of the Diabetic Rats

a) Acetone-Insoluble Lipids

Tissues	Saturated Fatty Acids Extracted		Unsaturated Fatty Acids Extracted		Rate of Incorporation into Unsaturated Acid
	Amount	Total Activity	Amount	Total Activity	
	mg.	c. p. m.	mg.	c. p. m.	%
Liver	3.3	1,255	7.1	732	37
Brain	3.0	890	4.2	138	13
Kidney	2.9	261	3.6	195	43
Intestine	0.9	1,593	2.4	1,422	47

b) Acetone-Soluble Lipids

Liver	3.8	775	3.9	739	49
Brain	0.4	30	0.9	61	—
Kidney	0.9	382	7.1	246	39
Intestine	3.7	5,775	10.0	7,050	55

The experimental conditions are described in Table I.

TABLE III

Desaturation of Palmitic Acid-1-C¹⁴ by Rat Liver Preparations

Rat liver preparations (15 mg. of protein) were incubated with 15 μ moles of palmitic acid-1-C¹⁴ (10,000 c. p. m.), 10 μ moles of ATP, 1 mmole of glucose and Krebs-Ringer phosphate buffer (pH 7.4) in a final volume of 8 ml., in air for 3 hours at 37°C.

Preparations	Fatty Acids Extracted			Desaturation ²⁾
	Saturated (a)	Unsaturated (b)	Total ¹⁾ (c)	
	c. p. m.	c. p. m.	c. p. m.	%
Mitochondria	2,860	620	3,480	18
Microsomes ³⁾	2,680	920	3,600	27
Mitochondria Microsomes ³⁾	2,450	995	3,455	29

1) Radioactivity of the total fatty acids extracted (c) were calculated as (a)+(b).

2) The desaturation grade was calculated as $100 \times (b)/(c)$.

3) Microsome fraction contained also supernatant fraction.

The synthesis of fatty acids in tissues was studied by comparing the labeling of these acids. As the components of compound lipid (Table I-a), long chain fatty acids were also synthesized from acetate. The rate of radioincorporation into unsaturated fatty acid from acetate was calculated, whereby the isotopic label in unsaturated fatty acid was compared with that in the whole fatty acid using total

radioactivity as indicator. The rate of radioincorporation into unsaturated acids was highest in testis (70 per cent), and lowest in intestine (46 per cent).

The fatty acid residue of acetone-soluble lipid (Table I-b), both saturated and unsaturated acids, were also synthesized from acetate. The rate of formation of unsaturated acids was highest in testis (66 per cent) and

TABLE IV

Cofactor Requirements and Effect of Oxygen for Desaturation of Palmitic Acid

Complete system: Flasks contained rat liver microsomes and supernate (10 mg. of protein), 15 μ moles of palmitic acid-1-C¹⁴ (10,000 c. p. m.), 1 μ mole of TPN, 10 μ moles of ATP, 1 mmole of glucose and Krebs-Ringer phosphate buffer (9), pH 7.4, in a final volume of 8 ml. After aeration either with O₂ or with N₂, incubation was carried out for 3 hours at 37°C.

Preparation	Gas Phase	Fatty Acids		Desaturation
		Total	Unsaturated	
		c. p. m.	c. p. m.	
Complete system	O ₂	2,520	985	39
"	N ₂	1,570	190	12
-ATP	O ₂	1,360	150	11
-TPN	O ₂	2,010	265	13
-ATP, -TPN	O ₂	1,780	160	9
-ATP, -TPN	N ₂	3,565	245	7
Control (boiled enzyme)	O ₂	2,600	85	3

lowest in lung (39 percent). Under the aerobic condition, therefore, both saturated and unsaturated fatty acids were formed from acetate almost to the same extent.

As shown in Table II, the labeling of long chain fatty acids from acetate-1-C¹⁴ was depressed in various tissue slices of diabetic rats, even though the amount of each acid was increased. The labeling of saturated or unsaturated fatty acid from acetate-C¹⁴ was compared with that of controls in each tissue. A decrease of radioincorporation into saturated and unsaturated acids was observed.

Desaturation of Palmitate—The greater enzymatic activity with regard to the desaturation of palmitic acid-1-C¹⁴ was found in the microsomal and supernatant fractions than in the mitochondrial fraction alone (Table III).

The requirement of cofactor in the desaturation of palmitic acid-1-C¹⁴ was studied. As shown in Table IV, the necessity of ATP and TPN were observed. When oxygen was replaced by nitrogen in the system, desaturation of palmitate decreased to a considerable extent.

The formation of monounsaturated fatty acid in liver preparations from diabetic and fasted rats was next investigated, by comparing the rate of desaturation of palmitate in

them with that in normal liver preparations. The fifteen liver preparations from normal rats showed the mean value of 27 per cent, whereas those in diabetic and fasted group were 16 and 14 per cent respectively (Table V), showing a definite decrease in the rate of desaturation in diabetic and fasted rats livers.

TABLE V

Desaturation of Palmitate by Liver Preparations of Diabetic and Starved Rats

The conditions of incubation were similar to those described in Table IV, except that TPN was replaced by a larger amount of liver preparations (40 to 50 mg. of protein). Gas phase was 95% oxygen.

Conditions	Fatty Acids Extracted		Desaturation
	Total	Unsaturated	
	c. p. m.	c. p. m.	
Normal	3,840	1,040	27
Diabetic	4,260	730	16
Starved	3,170	430	14

The data are expressed as the mean value of each 15 experiments.

DISCUSSION

The above experiments confirmed that the formation of long chain unsaturated fatty acid in rats required molecular oxygen.

Bernhard *et al.* (15) observed a conversion of stearic to oleic acid in a microsomal system of rat liver under an aerobic condition, and it was ascertained that lowered oxygen supply reduced *in vitro* lipogenesis, especially triglyceride synthesis from acetate (14). These findings can be explained by the necessity of oxygen for the formation of unsaturated fatty acids.

Synthesis *de novo* of long chain fatty acids from acetate was reduced in the alloxan diabetic animal. Both saturated and unsaturated fatty acids were affected. The formation of monounsaturated fatty acid from palmitate was also depressed in liver preparations from the diabetic as well as the fasted animals.

ATP and TPN were required for the desaturation of palmitate in the microsomal and supernatant fractions of normal rat liver. The required triphosphopyridine nucleotide participated in the reaction possibly as the reduced form, since both glucose-6-phosphate and 6-phosphogluconic dehydrogenases in the supernatant fraction of liver homogenates might generate the reduced coenzyme from the applied nucleotide. Furthermore, TPNH is required generally for reductive synthetic reactions (16), and TPN has been shown to be present in living cells mostly as reduced form, unlike DPN (17, 18).

Therefore, as indicated by Bloomfield and Bloch (7) in their yeast experiment, the desaturation of palmitate in the rat would require oxygen and a reduced pyridine nucleotide.

As to the lipogenic defect in diabetes, Siperstein and Fagan (3) investigated the relative importance of two known glycolytic pathways, the Embden-Meyerhof route and the pentose phosphate pathway. They found that the former route of glycolysis, though more important for the standpoint of glucose catabolism, played a relatively minor role in control of lipogenesis.

Since it seems certain that TPN in reduced form is needed for the desaturation of fatty acid, as in the synthesis of fatty acid from acetate, the reduced availability of TPNH may be regarded as an important

factor in lipogenic defect in cases of diabetic and starvation.

SUMMARY

1. In various tissues of normal animals, both saturated and unsaturated fatty acids were formed from acetate almost to the same extent under aerobic conditions. The stimulatory effect of oxygen was observed in the desaturation of palmitate in microsomal and supernatant preparations of normal rat liver. ATP and TPN were required thereby.

2. Synthesis of long chain fatty acids, both of saturated and unsaturated, from acetate was depressed in various tissues of diabetic animal. Liver preparations from the diabetic as well as from the fasted rats were less active in desaturating palmitic acid than the controls.

The author wishes to express his deep gratitude to Prof. M. Yasuda under whose direction this work has been carried out.

REFERENCES

- (1) Stetten, D. Jr., and Boxer, G. E., *J. Biol. Chem.*, **156**, 271 (1944)
- (2) Masoro, E. J., Chaikoff, I. L., Chernick, S. S., and Felts, J. M., *J. Biol. Chem.*, **185**, 845, (1950)
- (3) Siperstein, M. D., and Fagan, V. M., *J. Clin. Invest.*, **37**, 1185 (1958)
- (4) Wong, K. L., and Van Bruggen, J. T., *J. Biol. Chem.*, **235**, 26 (1960)
- (5) Weiss, S. B., Kennedy, E. P., and Kiyasu, J. Y., *J. Biol. Chem.*, **235**, 40 (1960)
- (6) Andreasen, A. A., and Stier, T. J. B., *J. Cellular Comp. Physiol.*, **43**, 271 (1954)
- (7) Bloomfield, D. K., and Bloch, K., *J. Biol. Chem.*, **235**, 337 (1960)
- (8) Gore, M. B. R., and McIlwain, H., *J. Physiol.*, **117**, 471 (1952)
- (9) Bergström, S., and Pääbo, K., *Acta Chem. Scand.*, **8**, 1486 (1954)
- (10) Swern, D., Billen, G. N., Finfley, T. W., and Scanlan, J. T., *J. Am. Chem. Soc.*, **67**, 1786 (1945)
- (11) Swern, D., and Dickel, G. B., *J. Am. Chem. Soc.*, **76**, 1957 (1954)
- (12) Kasuga, S., Tsuchida, M., and Kushigemachi, A., *J. Japan. Biochem. Soc.*, **32**, 367 (1960)
- (13) Le Page, G. A., and Mueller, G. C., *J. Biol. Chem.*, **180**, 975 (1949)

- (14) Imai, Y., Takahashi, Y., Hachiya, K., and Ishizuka, Y., *Japan. J. Nutr.*, **18**, 87 (1960)
- (15) Bernhard, K., von Bulow-Koster, J., and Wagner, H., *Helv. Chim. Acta*, **42**, 152 (1959)
- (16) Horecker, B. L., and Hiatt, H. H., *New Engl. J. Med.*, **257**, 177, 225 (1958)
- (17) Glock, G. E., and McLean, P., *Biochem. J.*, **61**, 388 (1955)
- (18) Jacobson, K. B., and Kaplan, N. O., *J. Biol. Chem.*, **226**, 603 (1957)

The Fate of C^{14} -Labeled Dehydrocholic Acid in Guinea Pig

By MICHIO OGURA and KAZUMI YAMASAKI

(From the Department of Biochemistry, Tottori University School of Medicine,
Yonago, Tottori-ken)

(Received for publication, March 8, 1961)

About ten years ago, the late Prof. T. Shimizu (1) advanced an assumption about the biogenesis of bile acid, based upon the data accumulated during more than two decades since 1927. According to his assumption, the bile acid might find its origin in an unsaturated sterol such as 7-dehydrocholesterol or ergosterol and its first product might be cholic acid, which was transformed through the dehydrated form, say dehydrocholic acid, into the less hydroxylated bile acids. Thus, dehydrocholic acid was here regarded as a key intermediate in the formation of the bile acid.

On the basis of this assumption, a series of experiments have been carried out in this laboratory (2) to study quantitatively the fate of dehydrocholic acid administered to experimental animals, and since C^{14} -labeled bile acid became available (3), one of the most important data for this assumption has been examined with the use of labeled bile acid on rabbits (4-6), because a different pathway (7) of the bile acid biogenesis has recently become much more probable.

In this paper will be reported the metabolic fate of C^{14} -labeled dehydrocholic acid in guinea pigs. This experiment is actually a re-examination of those performed by Kim (8) and Sasaki (9), which should have afforded decisive evidences for this assumption.

EXPERIMENTALS

Dehydrocholic [$24-C^{14}$] acid was prepared by the method described in a previous report (5).

The bile acids used were prepared and purified until their melting points were in good agreement with those appearing in the literature.

Extraction of bile acids from biological materials collected, as well as their fractionation, was carried

out in the same way as described previously (5, 6).

The radioactivity of each sample of material was determined in most cases with a Geiger-Müller counter, and in case of the isotope dilution method, with a 4π -gas flow counter unless otherwise stated.

Two male guinea pigs weighing about 380 g. were injected intraperitoneally with C^{14} -labeled dehydrocholic acid dissolved in physiological saline as sodium salt (100 mg./kg. body weight; total radioactivity, 215,600 c.p.m.). They were kept together in a cage for the following 3 days and fed on vegetables as usual. Urine (without catheterization) and feces were collected daily during the experiment, and bladder bile and intestinal contents were obtained at the end of the experiment, when the animals were sacrificed.

RESULTS

I. Recovery of Radioisotope in Excreta and Intestinal Contents

Acidified urine and bile samples were each extracted with ether and then butanol; feces and intestinal contents were likewise treated after lipid was removed (5, 6). The ether and butanol extracts obtained from the excreta collected after administration of C^{14} -labeled dehydrocholic acid showed radioactivity recovery as summarized in Table I. About one-quarter of the administered isotope was recovered from all the excreta and intestinal contents collected and the excretion of isotope in urine continued for 3 days with rapid decrease.

II. Analysis of Urine Samples

The butanol extract obtained from each day's urine sample (1-3 day samples) was hydrolyzed with aqueous *N* potassium hydroxide solution at 40°C for 3 hours. The hydrolyzate was acidified with concentrated hydrochloric acid and extracted with ether.

TABLE I
Recovery of Radioisotope of the Material Collected

Material		Ether extract		Butanol extract		Total	
		c.p.m.	%	c.p.m.	%	c.p.m.	%
Urine	1st day	32,500	15.1	2,350	1.1	34,850	16.2
	2nd day	4,970	2.3	395	0.2	5,365	2.5
	3rd day	740	0.3	0	0.0	740	0.3
(total)						(40,955)	(19.0)
Bladder bile		1,860	0.9	3,090	1.4	4,950	2.3
Feces		4,660	2.2	95	0.0	4,755	2.2
Intestinal contents		4,980	2.3	585	0.3	5,565	2.6
Total						56,225	26.1

Materials were obtained from two guinea pigs to which C^{14} -labeled dehydrocholic acid (total activity: 215,600 c.p.m.) was administered.

Each extract thus obtained was combined with the above-mentioned ether extract of the respective urine samples.

The combined extract of each day's urine sample was then subjected to reversed-phase chromatography. By titration as well as by radioactivity determination of each 2 ml. of

appearing in each of them. The peaks were designated as Peaks $I_1 \sim III_1$ for the first day's urine sample, Peaks $I_2 \sim III_2$ for the second, and Peaks $I_3 \sim III_3$ for the third, respectively. Fig. 1 illustrates the chromatogram of the extract of the first day's urine.

Characterization of the Peaks: Peak I—The

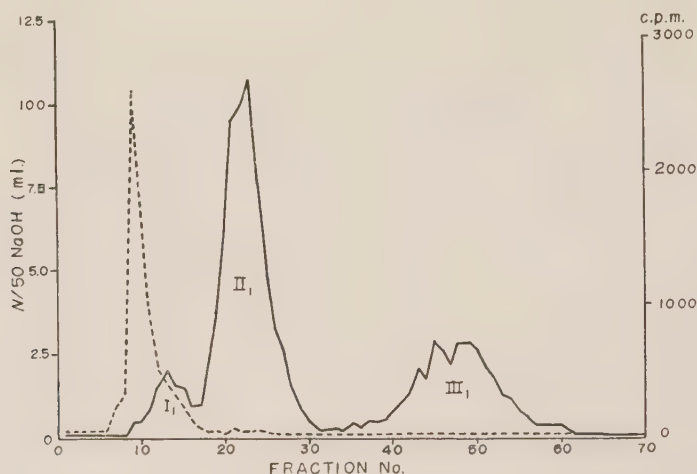


FIG. 1. Chromatogram of the extract of the first day's urine.

Stationary phase: Chloroform: heptane (9:1) supported on 4.5 g. of hydrophobic Celite. Moving phase: 60 per cent aqueous methanol.

— Radioactivity curve, --- Titration curve (Fig. 1-14)

the eluate, there were obtained three chromatograms corresponding to the urine samples of the 3 days, which were very similar in shape to each other, three radioactive peaks

eluates corresponding to Peak I_{1-3} were combined in aqueous solution, acidified with dilute hydrochloric acid, and extracted with ether. The ether solution was subjected to

steam distillation for 1 hour. The residual aqueous mixture was extracted with ether and the ether extract was washed, dried, and evaporated. The residue was again chromatographed. One peak was found in the radioactivity curve, as shown in Fig. 2.

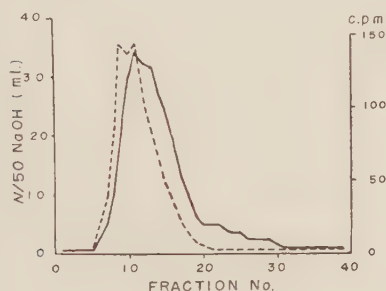


FIG. 2. Chromatogram of the extract corresponding to Peak I_{1-3} .
System: see Fig. 1.

The eluates corresponding to the peak were combined in aqueous solution, acidified, and extracted with ether. To this extract, 60 mg. of unlabeled cholic acid was added and the solvent was evaporated. The residue was recrystallized twice from methanol-ethyl acetate. Specific radioactivity of cholic acid remained unchanged after further recrystallization. The results were indicative of the presence of radioactive cholic acid in Peak I_{1-3} .

	Weight of crystals	Recrystn. solvent	c.p.m./mg.
1.	38 (mg.)	—	72
2.	32	ethyl acetate	75
3.	27	methanol-water	72

Peak II—The eluates corresponding to Peak II_1 (Fig. 1) were combined in aqueous solution, acidified, and extracted with ether. The ether solution was evaporated to dryness and chromatographed with 10 mg. of unlabeled 3α -hydroxy-7, 12-diketocholeic acid. On chromatography, only one peak was found in the radioactivity as well as in the titration curves for each, and these two were quite coincident with each other (Fig. 3).

Similar results were obtained with each extract of the eluates corresponding to Peaks II_2 and II_3 as with that of Peak II_1 . Hence,



FIG. 3. Chromatogram of the extract corresponding to Peak II_1 , with unlabeled 3α -hydroxy-7, 12-diketocholeic acid.
System: see Fig. 1.

the ether extracts obtained from the eluates corresponding to Peak II_{1-3} were combined and analyzed as follows: To the ether solution 30 mg. of unlabeled 3β -hydroxy-7, 12-diketocholeic acid (obtained from toad urine (8)) was added and the solvent was evaporated. The residue was recrystallized twice from methanol-water. Specific radioactivity of the crystals, melting at $256\sim 257^\circ\text{C}$, remained constant during repeated recrystallizations. The results clearly indicated that radioactive 3β -hydroxy-7, 12-diketocholeic acid was contained in Peak II.

	Weight of crystals	Recrystn. solvent	c.p.m./mg. ¹⁾
1.	16 (mg.)	—	106
2.	10	methanol-water	103
3.	7	//	107

1) Geiger-Müller counter

The mother liquor, from which the first crop of the above β -acid was separated, was mixed with 70 mg. of unlabeled 3α -hydroxy-7, 12-diketocholeic acid and the solvent was evaporated to dryness. The residue was recrystallized twice from methanol-water and the resulting crystals (m.p. $189\sim 190^\circ\text{C}$) were subjected to further recrystallization, radioactivity of each crop being determined. The results were as follows:

	Weight of crystals	Recrystn. solvent	c.p.m./mg. ¹⁾
1.	74 (mg.)	—	35
2.	69	methanol-water	41
3.	56	"	36
4.	44	"	38

1) Geiger-Müller counter

It was obvious enough from these results that Peak II contained two radioactive acids, i.e., 3β - and 3α -hydroxy-7,12-diketocholanic acids.

Peak III—Eluates corresponding to Peaks III₁₋₃ were separately collected in aqueous solution, acidified, and extracted with ether. Ether extract of Peak III₁ was evaporated to dryness and chromatographed together with 5 mg. of unlabeled dehydrocholic acid. On chromatography, one peak was found in the radioactivity curve and it was quite coincident with the one appearing in the titration curve, due to the unlabeled dehydrocholic acid added (Fig. 4).

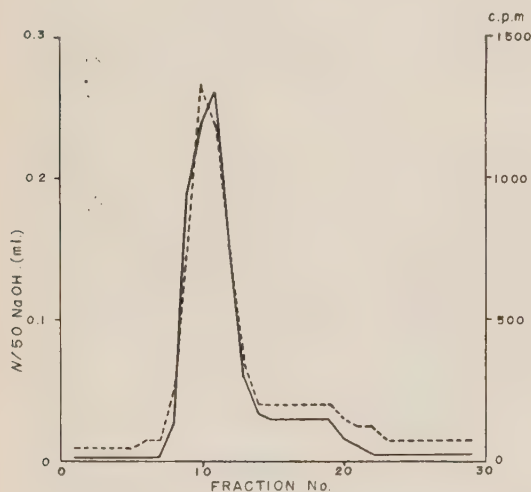


FIG. 4. Chromatogram of the extract corresponding to Peak III₁, with unlabeled dehydrocholic acid.

Stationary phase: Chloroform: heptane (9:1) supported on 4.5 g. of hydrophobic Celite.
Moving phase: 65 per cent aqueous methanol.

Accordingly, the eluates corresponding to Peak III₁₋₃ were combined in aqueous solution, acidified, and extracted with ether. To the ether solution 50 mg. of unlabeled dehydrocholic acid was added and the solvent was

evaporated. The residue was then recrystallized twice from methanol-water. Each crop of crystals from further recrystallization was proved to have a constant value of specific radioactivity. The results clearly showed that Peak III consisted of unchanged C¹⁴-labeled dehydrocholic acid.

	Weight of crystals	Recrystn. solvent	c.p.m./mg. ¹⁾
1.	32 (mg.)	—	53
2.	22	methanol-water	58
3.	12	"	57

1) Geiger-Müller counter

III. Analysis of Bile Samples

1. Analysis of the Ether Extract—The ether extract obtained from the bladder bile was chromatographed together with 5 mg. of deoxycholic acid. There was found one peak in each of the titration and radioactivity curves, the radioactivity peak lying in a more polar region than the titration one, due to the added deoxycholic acid (Fig. 5).

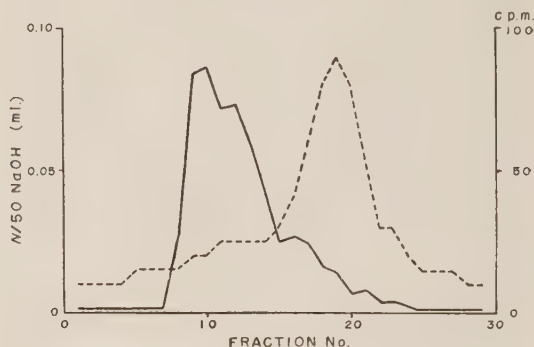


FIG. 5. Chromatogram of the ether extract of bladder bile.

System: see Fig. 1.

The eluates corresponding to the radioactive peak were collected in aqueous solution, acidified, and extracted with ether. To the ether solution, 50 mg. of unlabeled cholic acid was added and the solvent was evaporated to dryness. The residue was recrystallized from methanol-ethyl acetate mixture. The crystals thus obtained rapidly lost their radioactivity and completely after few recrystallizations from the same solvent.

The filtrates obtained at each step of

recrystallizations mentioned above were then combined and evaporated to dryness. The residue dissolved in 2 ml. of glacial acetic acid was treated with an excess of 1 per cent chromic acid solution (glacial acetic acid: sulfuric acid diluted with water (1:2)=9:1). After five minutes' standing at room temperature, the reaction mixture was diluted with water and extracted with ether. The ether extract was washed, dried, and evaporated. The residue was mixed with 30 mg. of dehydrocholic acid and recrystallized from methanol-water. It was found that radioactivity of the derived dehydrocholic acid was completely lost on repeated recrystallization.

Thus, although it was not possible to identify the radioactive substance contained in the bile as free bile acid, the data indicated that this substance was more polar than deoxycholic acid, but was neither cholic acid nor its stereoisomer.

2. *Analysis of the Butanol Extract*—The butanol extract obtained from the bladder bile was likewise analyzed chromatographically. In the radioactivity curve, there was found only one peak, which was quite coincident with the one appearing in the titration curve (Fig. 6).

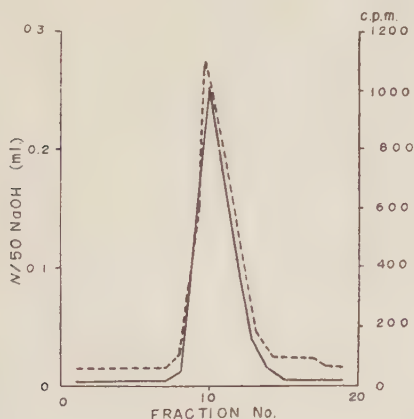


FIG. 6. Chromatogram of the butanol extract of bladder bile.

System: see Fig. 1.

The eluates corresponding to the peak were collected in aqueous solution and hydrolyzed with aqueous *N* potassium hydroxide

solution at 140°C for 3 hours. The hydrolyzate was shaken once with ether and the aqueous layer was separated. It was acidified with concentrated hydrochloric acid, and extracted with ether. The ether extract was washed, dried, and evaporated. The residue was again chromatographed. Four peaks were here found in the radioactivity curve and designated as Peaks IV, V, VI, and VII in the descending order (Fig. 7).

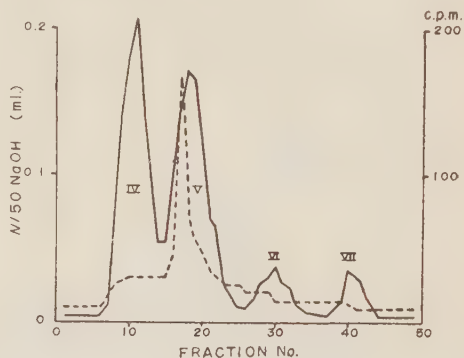


FIG. 7. Chromatogram of the hydrolyzate of the butanol extract.

System: see Fig. 1.

The eluates corresponding to each of the peaks were collected in aqueous solution, acidified, and extracted with ether. The solvent was evaporated from the extract. Each fraction so obtained was separately analyzed.

Characterization of the Peaks: Peak IV—The extract corresponding to Peak IV was chromatographed with addition of 10 mg. of unlabeled cholic acid. One peak appeared in each of the titration and radioactivity curve, and both of them were quite coincident with each other (Fig. 8).

The eluates corresponding to the peak were collected, acidified, and extracted with ether. To the ether extract further 70 mg. of unlabeled cholic acid was added, and ether was evaporated. The residue was recrystallized twice from methanol-ethyl acetate mixture. Each crop of cholic acid was found to have a constant value of specific radioactivity, when further recrystallization was conducted. It was therefore demonstrated that Peak IV consisted of radioactive cholic acid.

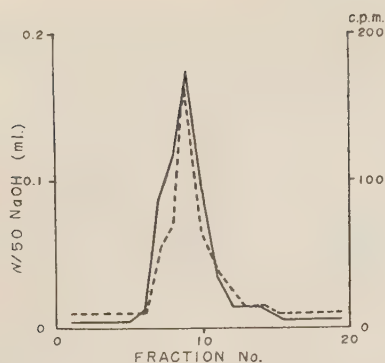


FIG. 8. Chromatogram of the extract corresponding to Peak IV, with unlabeled cholic acid. System: see Fig. 1.

	Weight of crystals	Recrystn. solvent	c.p.m./mg.
1.	51 (mg.)	—	73
2.	47	methanol-ethyl acetate	78
3.	31	ethyl acetate	77
4.	19	methanol-water	75

Peak V—The extract corresponding to Peak V was chromatographed together with 10 mg. of unlabeled deoxycholic acid. One peak was found in each of the radioactivity and titration curves, these two being exactly coincident with each other (Fig. 9).

was evaporated. The two parts were treated in different ways as follows: One part was mixed with 60 mg. of unlabeled deoxycholic acid and recrystallized twice from acetic acid, affording crystals of m.p. 144~145°C, with a constant value of specific activity on repeated recrystallization. It became quite clear from these results that radioactive deoxycholic acid was present in Peak V.

	Weight of crystals	Recrystn. solvent	c.p.m./mg.
1.	37 (mg.)	—	70
2.	29	acetic acid	78
3.	21	//	75
4.	16	//	73

The other part of the extract was mixed with 80 mg. of chenodeoxycholic acid and converted into formyl ester (dissolved in 2 ml. of formic acid and heated at 60°C for 5.5 hours). The reaction mixture was diluted with water and extracted with ether. The ether extract was washed, dried, and evaporated to dryness. The residue was recrystallized twice from ethanol-water. The crystals of diformylchenodeoxycholic acid, melting at 180~181°C, rapidly lost their radioactivity on recrystallization. The result was indicative of radioactive chenodeoxycholic acid being

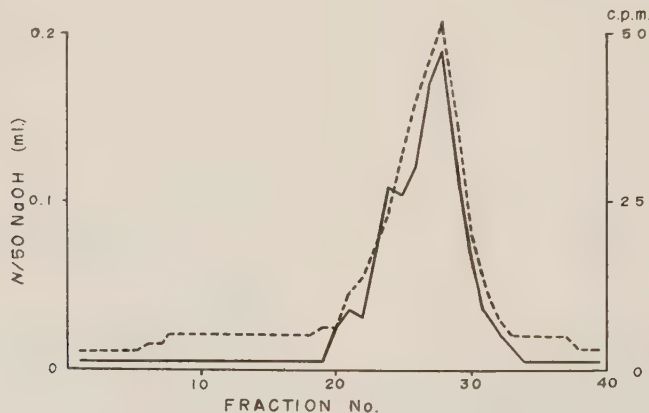


FIG. 9. Chromatogram of the extract corresponding to Peak V, with unlabeled deoxycholic acid. System: see Fig. 1.

The eluates corresponding to the peak were collected, acidified, and extracted with ether. The ether solution thus obtained was divided into two equal parts and the solvent

absent in Peak V.

Peak VI—The extract corresponding to Peak VI was chromatographed together with 10 mg. of 3 α -hydroxy-7-ketocholanic acid. In

the titration and radioactivity curves, one peak was found for each; the radioactive peak stood in a region less polar than the titration one (Fig. 10).

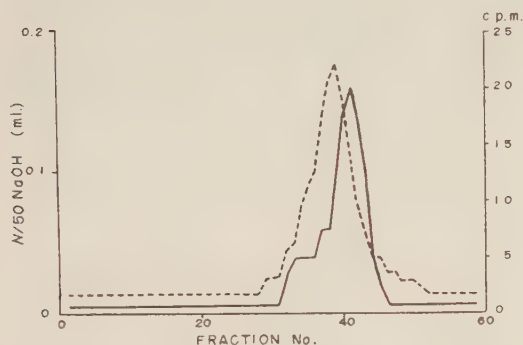


FIG. 10. Chromatogram of the extract corresponding to Peak VI, with unlabeled 3 α -hydroxy-7-ketocholanic acid.

System: see Fig. 1.

To the eluates corresponding to the radioactive peak 50 mg. of 3 α -hydroxy-12-ketocholanic acid was added, the mixture was recrystallized repeatedly from methanol-water, and the radioactivity was completely lost.

All the filtrates of the above recrystallizations were then combined and evaporated to dryness. The residue dissolved in 2 ml. of acetic acid was oxidized with chromic acid as described above. The reaction mixture was diluted with water and extracted with ether. The ether extract was washed, dried, and evaporated. To the residue 20 mg. of 3, 12-diketocholanic acid was added and it was recrystallized twice from acetone-water. The crystals of 3, 12-diketocholanic acid had a constant value of specific radioactivity on further recrystallization. Thus, it became clear that the radioactive compound contained in Peak VI was identical neither with 3 α -hydroxy-7-ketocholanic nor with 3 α -hydroxy-12-ketocholanic acid. It may be an isomer of the latter, probably 3 β -hydroxy-12-ketocholanic acid.

	Weight of crystals	Recrystn. solvent	c.p.m./mg.
1.	13 (mg.)	—	24
2.	6	acetone-water	24
3.	2	//	28

Peak VII—The extract corresponding to Peak VII was chromatographed together with 10 mg. of unlabeled lithocholic acid. In the radioactivity curve were found four peaks appearing around the one peak in the titration curve, due to lithocholic acid added (Fig. 11). Owing to poor activity of these peaks, it was not possible to identify any of these radioactive compounds.

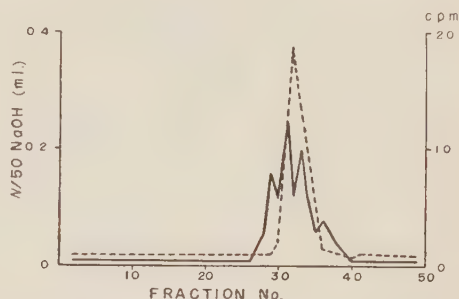


FIG. 11. Chromatogram of the extract corresponding to Peak VII, with unlabeled lithocholic acid.

System: see Fig. 1.

IV. Analysis of Feces and Intestinal Contents

The ether and butanol extracts obtained from the feces and intestinal contents collected were combined and hydrolyzed with aqueous *N* potassium hydroxide solution at 140°C for 3 hours. The alkaline hydrolyzate was shaken once with ether and the aqueous layer was separated. The aqueous solution was acidified with concentrated hydrochloric acid

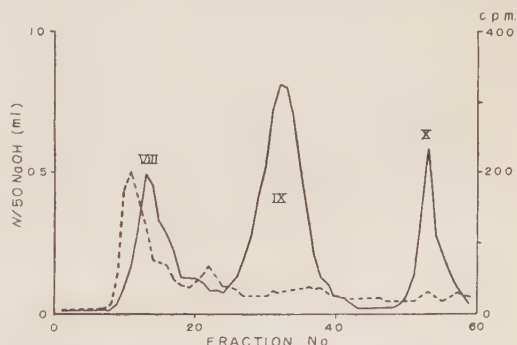


FIG. 12. Chromatogram of the extract of feces and intestinal contents.

System: see Fig. 1.

and extracted with ether. The ether solution was subjected to steam distillation for 1 hour. The residual aqueous mixture was extracted with ether after addition of dilute hydrochloric acid, the solvent was evaporated, and the residue was chromatographed. Three peaks were found in the radioactivity curve and were designated as Peaks VIII, IX, and X in the order of elution (Fig. 12).

The eluates corresponding to each of the peaks were collected in aqueous solution, acidified, extracted with ether, and the solvent was evaporated from the extract.

Characterization of the Peaks: Peak VIII—The extract corresponding to Peak VIII was dissolved in acetone together with 120 mg. of cholic acid, the solution was divided into two equal parts, each of which was evaporated to dryness, and the two halves were treated in different ways.

One half was recrystallized several times from methanol-ethyl acetate and afforded crystals of cholic acid, specific activity of which remained constant on further recrystallization.

	Weight of crystals	Recrystn. solvent	c.p.m./mg.
1.	20 (mg.)	—	13
2.	15	ethyl acetate	12
3.	11	methanol-water	13

The other half of the extract dissolved in 2 ml. of acetic acid was oxidized with chromic acid in the same way as described above. The reaction mixture was diluted with water and extracted with ether. The solution was washed, dried, and evaporated. The residue was then recrystallized twice from methanol-water. The crystals, consisting mainly of dehydrocholic acid derived from the cholic acid added, melted at 235~236°C (mixed m.p. 236°C) and had a constant value of specific radioactivity by further recrystallization:

	Weight of crystals	Recrystn. solvent	c.p.m./mg.
1.	33 (mg.)	—	27
2.	25	methanol-water	25
3.	19	"	26
4.	15	"	27

From these experiments it was clearly demonstrated that radioactive cholic acid was responsible for Peak VIII. It is interesting

to note, however, that specific radioactivity of the derived dehydrocholic acid was twice as high as that of the cholic acid found as itself. This finding strongly suggests that some kind of stereoisomer, say 3 β -isomer, of cholic acid was also contained in Peak VIII.

Peak IX—The extract corresponding to Peak IX was chromatographed together with 10 mg. of unlabeled deoxycholic acid. A peak appearing in the radioactivity curve was quite coincident with the one in the titration curve (Fig. 13).

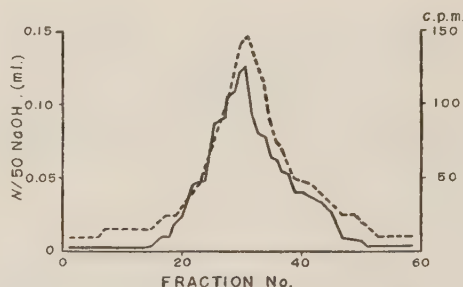


FIG. 13. Chromatogram of the extract corresponding to Peak IX, with unlabeled deoxycholic acid.

System: see Fig. 1.

The eluates corresponding to the peak were collected and extracted again with ether. The ether solution was then divided into two equal parts and treated in two different ways as follows: One part of the extract was mixed with 60 mg. of unlabeled deoxycholic acid and recrystallized twice from acetic acid. Specific radioactivity of the resulting acetocholenic acid maintained a constant value when recrystallization was repeated further.

	Weight of crystals	Recrystn. solvent	c.p.m./mg.
1.	41 (mg.)	—	192
2.	31	acetic acid	193
3.	27	"	187
4.	23	"	195

The other part of the extract was mixed with 60 mg. of unlabeled chenodeoxycholic acid and formylated in the same way as described above. It was found that the isolated diformylchenodeoxycholic acid became freed from radioactivity on repeated recrystallization from ethanol-water. Peak IX was proved

therefore to consist of deoxycholic acid, but not of chenodeoxycholic acid.

Peak X—The extract corresponding to Peak X was chromatographed together with 5 mg. of unlabeled lithocholic acid. One peak was found in each of the titration and radioactivity curves, but the radioactivity peak was located in a region more polar than the other (Fig. 14).

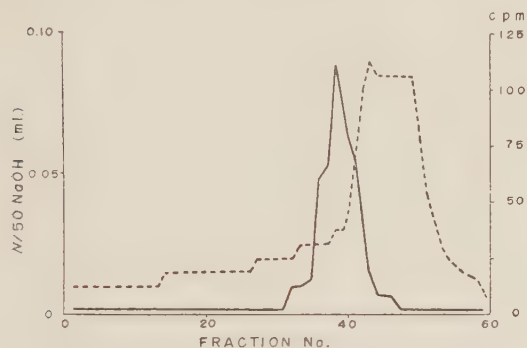


FIG. 14. Chromatogram of the extract corresponding to Peak X, with unlabeled lithocholic acid.

System: see Fig. 1.

The eluates corresponding to the radioactive peak were collected in aqueous solution, acidified, extracted with ether, and the solvent was evaporated from the extract. The residue was mixed with 60 mg. of unlabeled 3 α -hydroxy-12-ketocholanic acid and recrystallized from ethanol-water. Radioactivity of the crystals was lost completely after two recrystallizations from the same solvent. Hence, the mother liquor of each recrystallization was combined, mixed with more 70 mg. of 3 α -hydroxy-12-ketocholanic acid, and evaporated to dryness. The residue was dissolved in acetic acid and oxidized with chromic acid as described above. The reaction mixture was diluted with water, extracted with ether, and the solvent was evaporated from the extract. The residue was recrystallized twice from methanol-water. The crystals of the isolated 3,12-diketocholanic acid, melting at 188~189°C, had a constant value of specific activity on repeated recrystallization.

	Weight of crystals	Recrystn. solvent	c.p.m./mg.
1.	42 (mg.)	—	67

2.	33	methanol-water	76
3.	11	//	68
4.	6	//	70

The results indicated that Peak X did not contain radioactive 3 α -hydroxy-12-ketocholanic acid, but a stereoisomer of this acid, such as 3 β -hydroxy-12-ketocholanic acid, as described in the case of the bladder bile.

DISCUSSION

In order to prove Shimizu's assumption mentioned earlier Kim (9) and Sasaki (10) administered cholic, 3 α -hydroxy-7,12-diketocholanic, or dehydrocholic acid to guinea pigs, taking advantage of the fact that the bile of this animal species contains only chenodeoxycholic acid together with its partially oxidized acid (11), and that neither deoxycholic nor cholic acid is present naturally. They were able to isolate deoxycholic acid not only from urine but from bile of the animal and believed to have confirmed the assumption that cholic acid was converted in the animal organism into this bile acid, dehydrocholic acid being here inferred as an obligatory intermediate, and, furthermore, assumed that chenodeoxycholic acid proper

TABLE II

Metabolites of C¹⁴-Labeled Dehydrocholic Acid

Material	Metabolites identified
Urine	Cholic acid 3 α -Hydroxy-7, 12-diketocholanic acid 3 β -Hydroxy-7, 12-kiketocholanic acid Dehydrocholic acid
Bladder bile	Cholic acid Deoxycholic acid 3 β -Hydroxy-12-ketocholanic acid (identified as 3, 12-diketocholanic acid)
Feces and intestinal contents	Cholic acid 3 β -Isomer of cholic acid (identified as dehydrocholic acid) Deoxycholic acid 3 β -Hydroxy-12-ketocholanic acid (identified as 3, 12-diketocholanic acid)

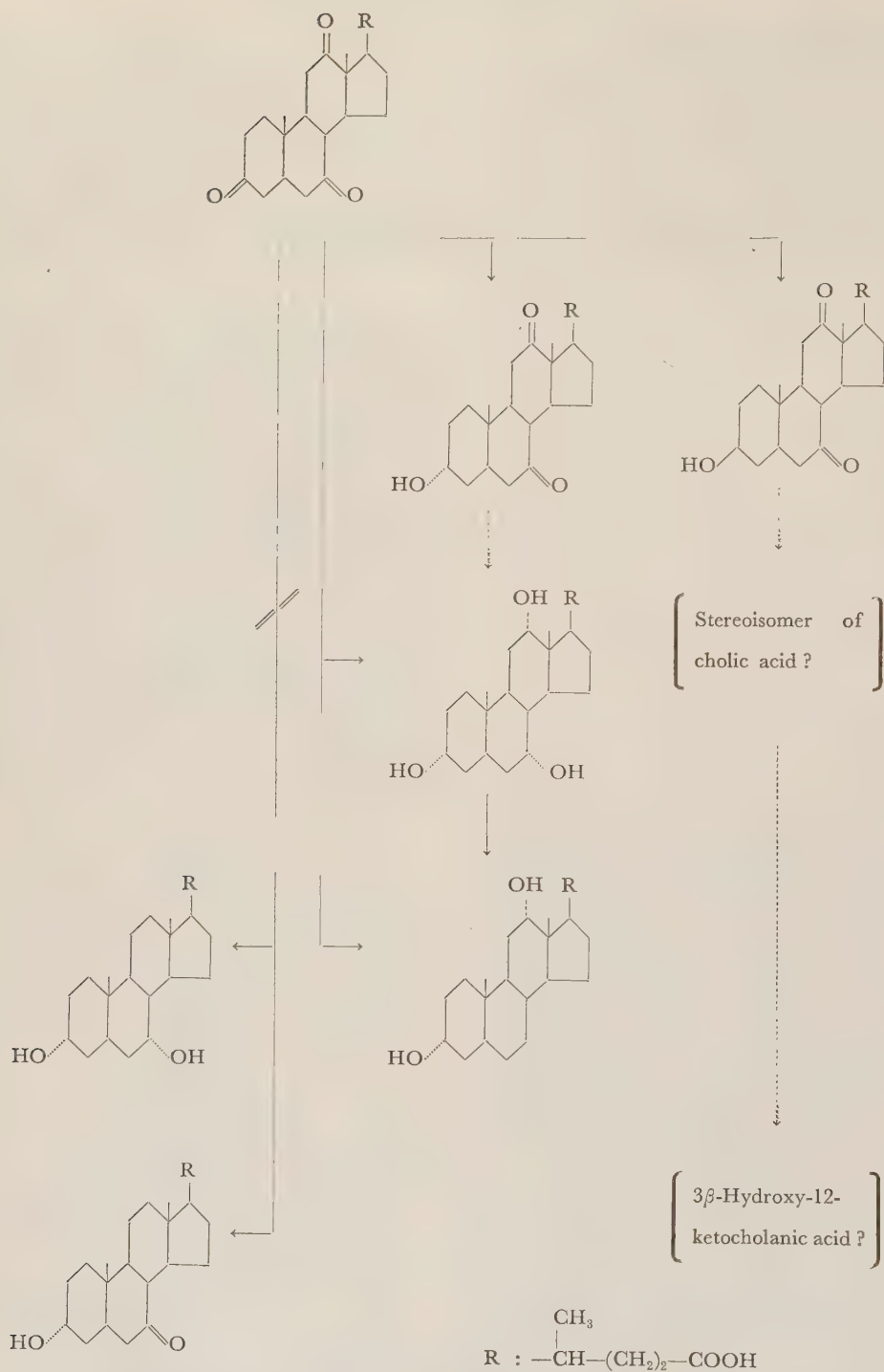


FIG. 15. Metabolic fate of dehydrocholic acid in guinea pig.
 Solid lines: Reactions that have been directly demonstrated. Broken lines: Probable pathways (not directly demonstrated).
 The reactions on the left side do not occur (\nrightarrow).

to this animal species was also derived from cholic acid, though not experimentally demonstrated.

The present results obtained (Table II) were satisfactorily consistent with the data of Kim (9) and Sasaki (10), and the metabolic fate of C¹⁴-labeled dehydrocholic acid administered to guinea pigs may be summarized as indicated in Fig. 15.

By the *in vivo* as well as *in vitro* experiments with rabbits or rats (6, 12, 13), it has become evident, that the liver is responsible for the reduction of dehydrocholic acid to 3 α - and/or 3 β -hydroxy-7,12-diketocholanic acid. The conclusive evidence, however, that further reduction of these hydroxydiketo bile acids to deoxycholic or chenodeoxycholic acid in the liver, according to Shimizu's assumption, has not yet been available. On the contrary, Bergström and his coworkers (7) have shown that cholic acid, during its enterohepatic circulation, is converted into deoxycholic acid through the action of the intestinal microorganisms and not by the liver enzyme, which was recently confirmed by the present workers (4).

The present experiment demonstrated that C¹⁴-labeled dehydrocholic acid found its way as C¹⁴-labeled deoxycholic acid in bile and feces, including intestinal contents. This finding might seemingly confirm Shimizu's assumption, but its interpretation must be changed, because C¹⁴-labeled deoxycholic acid could not be found in bile when C¹⁴-labeled dehydrocholic acid was injected after the animals were furnished with fistulas (4). This transformation is therefore attributed to the intestinal microorganisms and not to the liver enzyme system.

This interpretation was further confirmed by the fact that the isotope administered was not incorporated into any of the bile acids proper to guinea pig, such as chenodeoxycholic or 3 α -hydroxy-7-ketocholanic acid.

SUMMARY

1. Intact guinea pigs were administered intraperitoneally with sodium dehydrocholate

[24-C¹⁴], and labeled metabolites were traced in their excreta.

2. Radioactive bile acids found in excreta were as follows: Cholic, 3 α - and 3 β -hydroxy-7,12-diketocholanic, and dehydrocholic acids in urine; cholic and deoxycholic acids, and an isomer of 3 α -hydroxy-12-ketocholanic acid in bladder bile; cholic acid, its stereoisomer, deoxycholic acid, and an isomer of 3 α -hydroxy-12-ketocholanic acid in feces and intestinal contents.

3. The metabolic fate of dehydrocholic acid in guinea pig was discussed and Shimizu's assumption was criticized.

The authors express their thanks to Miss N. Hatanô for her technical assistance throughout this work.

The present work was aided by the Grant-in-Aid for Scientific Research from the Ministry of Education, to which the authors' gratitude is due.

REFERENCES

- (1) Shimizu, T., *J. Japan. Biochem. Soc.*, **20**, 118 (1948); "Über die Chemie u. Physiologie der Gallensäuren", M. Muramoto Insatsu Co., Okayama (1935)
- (2) Yamasaki, K., "Chemistry and Physiology of Bile Acids and the Pineal Body", Daigaku Insatsu Co., Hiroshima, pp. 17~40, (1960)
- (3) Bergström, S., Rottenberg, M., and Volts, I., *Acta Chem. Scand.*, **7**, 481 (1953)
- (4) Yamasaki, K., Wakutani, T., Takimoto, H., and Shimizu, K., *J. Biochem. (Tokyo)*, **46**, 809 (1959)
- (5) Yamasaki, K., Kawahara, T., *Yonago Acta Medica*, **4**, 40 (1959)
- (6) Ogura, M., Wakutani, T., and Yamasaki, K., *Yonago Acta Medica*, **4**, 179 (1960)
- (7) Bergström, S., Record of *Chem. Progr.*, **16**, 63 (1955); Lindstedt, S., and Sjövall, J., *Acta Chem. Scand.*, **11**, 421 (1957); Norman, A., and Sjövall, J., *J. Biol. Chem.*, **233**, 872 (1958)
- (8) Yamasaki, K., and Kyogoku, K., *Z. physiol. Chem.*, **235**, 43 (1935)
- (9) Kim, C.H., *Z. physiol. Chem.*, **261**, 97 (1939)
- (10) Sasaki, T., *J. Biochem. (Tokyo)*, **32**, 81; 87 (1940)
- (11) Imai, I., *Z. physiol. Chem.*, **248**, 65 (1937)
- (12) Ogura, M., *J. Biochem. (Tokyo)*, **46**, 1077 (1959)
- (13) Ogura, M., Kôno, G., and Yamasaki, K., *J. Yonago Med. Assoc. (Japanese)*, **11**, 149 (1960)

Studies on the Biosynthesis of Antibody and Other γ -Globulin in Microsomes of Immunized Spleen Cells

By KIKUO OGATA, SHOZO OMORI and RYOJI HIROKAWA

(From the Department of Biochemistry, Niigata University,
School of Medicine, Niigata)

(Received for publication, March 10, 1961)

In the previous report (1) from this laboratory it was demonstrated that the cell suspensions from the popliteal lymph nodes of rabbits, immunized by injection of crystalline ovalbumin at foot pads, had a very high activity to incorporate C^{14} -glycine into antibody under aerobic conditions. There are several works now available, which indicate the incorporation of labeled amino acids into antibody by immunized lymphatic and reticuloendothelial tissue slices (2-5). Since it is well known that antibody is generally closely associated with serum γ -globulin, it seems very reasonable to suppose the intimate correlation between the biosynthesis of these two proteins in the immunized animal. It was shown by the authors (6) that slices of lymphatic and reticuloendothelial tissues, especially those of spleen from immunized rabbits showed a high activity in the incorporation of C^{14} -glycine not only into antibody but also into other γ -globulin which is not precipitable by specific antigen but precipitable by antiserum against the rabbit serum γ -globulin. Similar results, indicating a high activity of the lymphatic and reticuloendothelial tissues of immunized rabbits in the incorporation of labeled amino acid into antibody and other γ -globulin *in vivo* and in slices, were recently reported by Askonas and others (7, 8). Askonas (9) further reported net synthesis of antibody by the isolated perfused lung of hyperimmunized rabbits. Simultaneous occurrence of net synthesis and labeling of antibody in spleen or lymph nodes cells *in vitro* were demonstrated by Steiner and Anker (10) Stavitsky and Wolf (11, 12) and La Via *et al.* (13), employing tissue culture technique which

allowed the cells to remain active for 1-3 days. Higashi and Hasegawa (14) reported the net synthesis of serum γ -globulin by normal horse liver slices.

Concerning the intracellular site of serum protein synthesis in animal tissues, Peters (15) first indicated from the results of the isotopic experiments *in vivo* and with liver slices that liver microsomes were the mainsite of albumin synthesis. Recently, Campbell *et al.* (16) and the authors (17, 18) succeeded in demonstrating the incorporation of C^{14} -amino acid into microsomal albumin by a cell free system consisting of liver microsomes and cell sap or the pH 5 fraction.

This communication describes the study on the intracellular site in spleen cells of immunized rabbits for the synthesis of antibody and other γ -globulin *in vivo* and in slices. The cell free system which is responsible for the incorporation of C^{14} -amino acid into the total γ -globulin in microsomes of hyperimmunized spleen cell is also reported.

EXPERIMENTAL

Chemicals— C^{14} -2-Glycine (1 μ c/0.258 μ mole), C^{14} -L-leucine (uniformly labeled, 1 μ c/0.169 μ mole), and an acid hydrolysate of algal C^{14} -protein (1 μ c/0.005 mg.) were obtained from Radiochemical Centre, Amersham, England. S^{35} -L-Methionine was prepared by the modified method of Niklas (19) and the specific radioactivity was 1 μ c/0.173 μ moles or 1 μ c/0.547 μ moles in use.

Adenosine triphosphate (disodium salt, ATP) and guanosine triphosphate (sodium salt, GTP) were obtained from Sigma Chemical Co., U. S. A. Phosphocreatine (sodium salt) was prepared by the method of Lardy *et al.* (20), and creatine kinase by that of Kuby *et al.* (21). Crystalline ribonuclease (RNase)

and sodium desoxycholate were obtained from Worthington Biochemical Co. and Difco Lab., respectively.

Immunological Procedures—Adult albino rabbits (about 2.5 kg) received intravenous injection of 10 mg. of alum-precipitated crystalline ovalbumin, purified by the method of Keckwick *et al.* (22) and recrystallized 3 times. The injection was made on alternative days for at least 4 weeks. The serum antibody level was measured 3 to 5 days after the last injection by the quantitative precipitin method of Heidelberger and Kendall (23) and animal with the high serum titer (above 0.8 mg. N of antigen-antibody precipitate per 1 ml. of serum) was used. At least 30 days after the last antigen injection, the rabbits received intravenous injection of 50–100 mg. of antigen at once. The hyperimmunized rabbits thus obtained were employed in the following experiments.

For the preparation of antiserum against rabbit γ -globulin, alum precipitated rabbit serum γ -globulin, purified 3 times by the ammonium sulphate fractionation (between 1.1 *M* and 1.35 *M*) and showing single component in electrophoretic or ultracentrifugal analysis, was given intravenously to cocks every other days for at least 4 weeks.

Fractionation of Cellular Components—The modified method of Hogeboom *et al.* (24) were employed. The spleen was homogenized in a Potter-Elvehjem type glass homogenizer with 3 volumes of 0.25 *M* sucrose in a cold room. Nuclear, mitochondrial, microsomal and supernatant fractions were isolated by means of differential centrifugation for 10 minutes at $700\times g$, for 10 minutes at $10,000\times g$ and for 90 minutes at $105,000\times g$, respectively. The nuclear fraction was further purified by the procedures described by Hogeboom *et al.* (24).

Fractionation of Antibody and γ -Globulin from Each Cellular Component—The particulate fractions were treated with 0.8% sodium desoxycholate at 0°C for 2 hours by the method of Peters (15) and then the suspension was centrifuged at $105,000\times g$ for 90 minutes. The desoxycholate soluble fraction thus obtained as well as cellular supernatant fraction was dialyzed against 0.9% saline adjusted to pH 7.6 with Tris (hydroxymethyl) aminomethane (Tris) buffer at 0°C for 24 hours.

After removing unspecific lipoprotein by the method of Campbell (25) the desoxycholate soluble, supernatant and medium fractions were adjusted to pH 4.8 with 0.1 *N* acetic acid, followed by centrifugation for 10 minutes at $10,000\times g$, in order to remove the postmicrosomal fraction (26) from the supernatant fraction and to avoid the possible contamination of unspecific proteins from antibody and γ -globulin fraction.* The clear supernatants thus obtained were neutral-

ized to pH 7.0 and then subjected to the isolation of antibody and other γ -globulin. After removing unspecific protein by adding bovine serum albumin and suitable amount of cock antiserum against bovine serum albumin (23), specific antibody was precipitated by addition of carrier antiserum (rabbit anti-ovalbumin serum) and suitable amount of antigen. After the isolation of antibody, other γ -globulin was fractionated by addition of a calculated amount of cock anti-rabbit γ -globulin serum (23). In the case of the fractionation of the total γ -globulin including antibody, the procedure of antibody isolation was omitted. The specific precipitates thus obtained (antibody and γ -globulin) were centrifuged and washed twice with ice cold 0.9% saline. The detailed procedures were shown in our previous papers (6).

Experiment in Vivo—The rabbit was injected intravenously with 20 μ c of C^{14} -glycine or 40 μ c of S^{35} -methionine 96 hours after the last antigen injection, since the antibody formation *in vivo* (27) and in spleen slices (6) was found to be most active at this time. At a given time after the injection of labeled amino acid, the rabbit was killed by exsanguination and the spleen was chilled and perfused rapidly with ice cold 0.9% saline in a cold room. The spleen was then subjected to cellular fractionation and the total γ -globulin of each cellular fraction was separated as described above.

Preparation and Incubation of Spleen Slices—The rabbit was killed by slowly bleeding from one of carotids, the spleen perfused and slices of about 0.5 mm. thickness were prepared with Stadie-type slicer in a cold room.

One gram of slices was incubated with 0.4 ml. (5 μ c) of C^{14} -glycine in Warburg vessel, containing 2.6 ml. of slightly modified Krebs-Ringer phosphate solution** as described in our previous paper (1), at 37°C under the atmosphere of oxygen. Immediately after the incubation, the vessels were chilled in ice cold water, and the contents were centrifuged for 10 minutes at $700\times g$. The following procedures were carried out in a cold room. The precipitated slices were then washed twice with 1 ml. of the cold medium by centrifugation. Each of supernatants was collected and used as the medium fraction, and the washed slices were subjected to the cellular fractionation.

Preparation and Incubation of Cell Free System—Spleen microsomes and the pH 5 fraction were prepared

* This procedure was usually omitted in the case of cell free experiments.

** 0.9% NaCl, 100; 1.15% KCl, 4; 3.84% $MgSO_4 \cdot 7H_2O$, 1; 0.1 *M* Sørensen's phosphate buffer (pH 7.8), 6; 1.22% $CaCl_2$, 3.

by the method of Keller and Zamecnik (28), except that 0.05 *M* Tris buffer (pH 7.6) was substituted for bicarbonate buffer as described by Littlefield and Keller (29), and that the pH 5 fraction was precipitated at pH 4.8 by adding 0.1 *N* acetic acid. The pH 5 supernatant fraction was prepared by neutralizing the supernatant after precipitation of the pH 5 fraction with 0.1 *N* KOH, and an enzyme fraction having an activity in transfer of C^{14} -amino acids from soluble RNA to microsomal protein was fractionated from the rat liver pH 5 supernatant fraction with ammonium sulphate between 30 and 50 per cent saturation and treated with the gel filtration method of Porath and Flodin (30). The soluble RNA labeled with C^{14} -amino acids was prepared from rat liver pH 5 fraction by the method of Hoagland *et al.* (31) employing an acid hydrolysate of algal C^{14} -protein.

The basic incubation medium contained in a total volume of 2 ml; 5 *mM* $MgCl_2$, 50 *mM* KCl , 10 *mM* $KHCO_3$, 50 *mM* Tris buffer (pH 7.9), 1 *mM* ATP, 0.25 *mM* GTP, 43 μM C^{14} -leucine (total counts, 6.2×10^5 counts per minute), 20 *mM* phosphocreatine, 100 $\mu g.$ of creatine kinase, about 8 mg. protein of spleen microsomes, and about 6 mg. protein of rat liver pH 5 fraction. The incubation was carried out at 30°C for 30 minutes.

The reaction was stopped by adding an ice cold medium containing 0.01% C^{12} -leucine or C^{12} -amino acids mixture and the reaction mixture was centrifuged at $105,000 \times g$ for 90 minutes. The microsomal fraction thus obtained was subjected to desoxycholate treatment as described above.

In the case of the transfer experiment, the soluble RNA labeled with C^{14} -amino acids and pH 5 supernatant fraction or the enzyme fraction were substituted for C^{14} -leucine and the pH 5 fraction respectively.

Washing and Determination of Radioactivity of Specific Precipitates and Other Protein Fractions—Each protein fraction was then treated with 5% hot trichloroacetic acid and the precipitate was washed 4 times with 5% trichloroacetic acid, 2 times with absolute ethanol and 2 times with hot ethanol-ether (3:1).

The protein fractions were then put in a stainless-steel or aluminium disk, dried and weighed, and their radioactivity was determined in a RIDL Model 21 preflush flow counter or a Lauritzen Electroscop and suitable self-absorption factors were applied to correct counts to zero thickness. The specific activity of the total γ -globulin of each cellular fraction was corrected by the factor for antigen-antibody ratio previously determined according to Heidelberger's method (23).*

Analytical—Protein was determined by the modified

method of Folin and Ciocalteu (32).

RESULTS

The purified rabbit serum γ -globulin and the extract from each cellular fraction of hyperimmunized spleen were tested for antigenic heterogeneity by Ouchterlony's gel diffusion method (33), employing cock antiserum against rabbit serum γ -globulin. Double diffusion in agar with purified serum γ -globulin and the extract of each cellular fraction gave one precipitation line and migrated completely. This indicates immunological identity of our γ -globulin fraction of each cellular component with the serum γ -globulin (Fig. 1).

The in Vivo Incorporation of Labeled Amino Acid into the Total γ -Globulin of Each Cellular Component of Hyperimmunized Rabbit Spleen—In order to investigate the relative rate of the incorporation of labeled amino acid into the total γ -globulin (including antibody) of each cellular component *in vivo*, the specific activity of the total γ -globulin of mitochondria, nuclei and supernatant was expressed as per cent of that of microsomes at given times up to 2 hours after the injection of C^{14} -glycine or S^{35} -methionine (Fig. 2). It was observed that the specific activity of the total γ -globulin of microsomes up to 60 minutes after injection was higher than that of other cellular fractions and at 2 hours the specific activity of the total γ -globulin of mitochondria became higher than that of microsomes. It was further found that the specific activity of the total γ -globulin of each cellular component was remarkably higher than that of serum antibody and other γ -globulin even at 2 hours after injection. The results may indicate that the synthesis of γ -globulin including antibody is highly active in microsomes and suggest the possibility that microsomal γ -globulin is the precursor of that of other cellular components although the synthetic activity of mitochondria and nuclei can not be completely ruled out.

* In this case the addition of carrier rabbit serum γ -globulin against ovalbumin was not carried out before the precipitation of γ -globulin as described above.

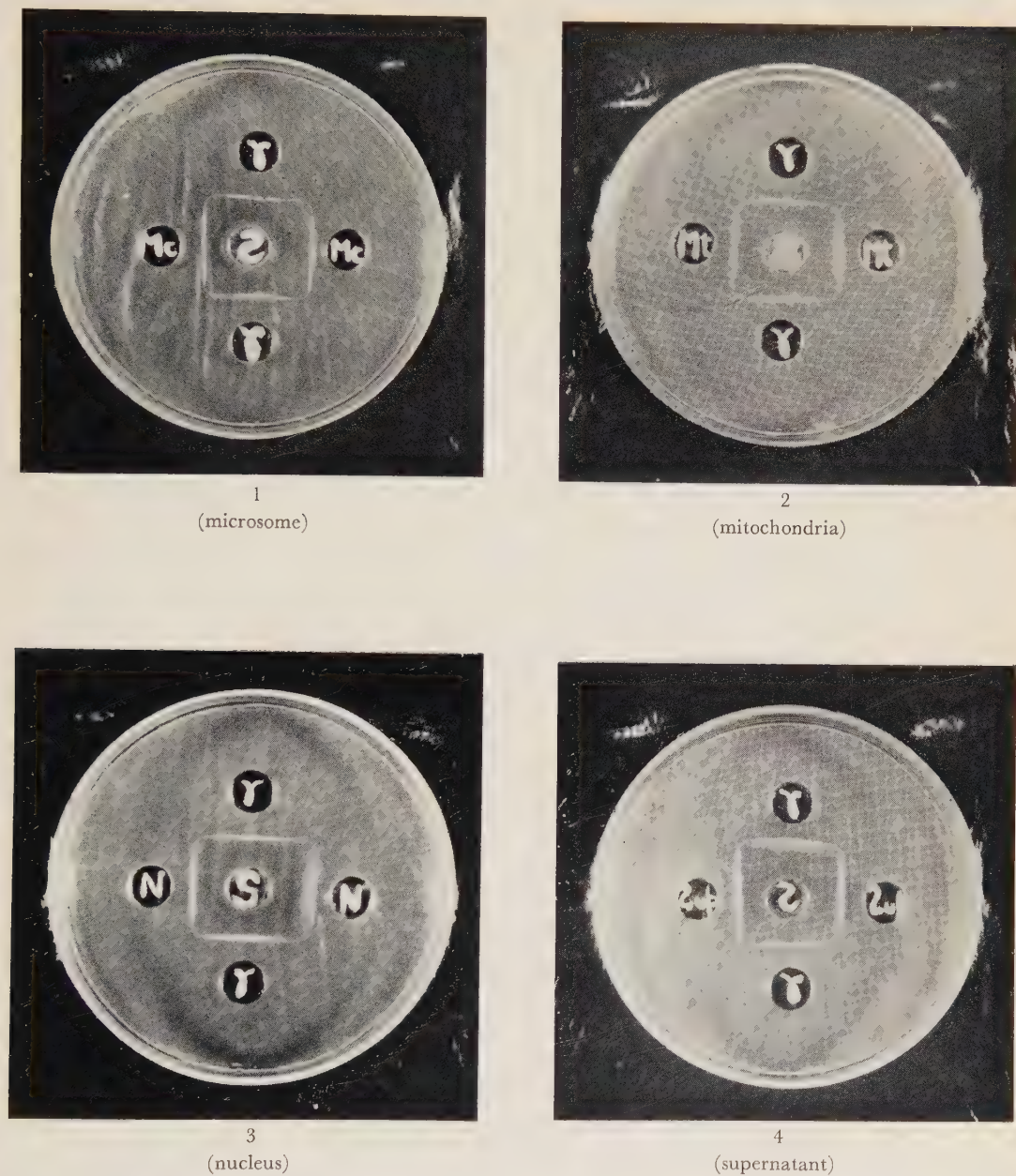


FIG. 1. Double diffusion in agar of rabbit spleen cellular fractions.

Center well: cock antiserum against rabbit serum γ -globulin; γ : γ -globulin; Mc, Mt, N: dialyzed desoxycholate extracts of microsome, mitochondria and nuclei respectively; Sup: dialyzed supernatant; diffusion time: 4 days (at 37°C)

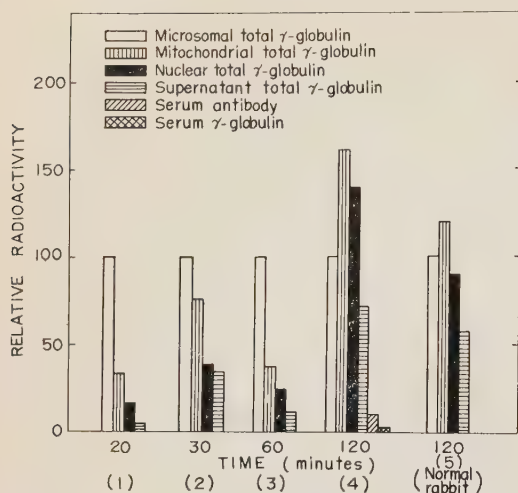
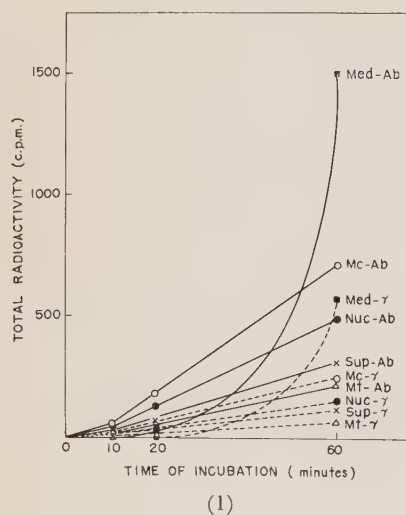
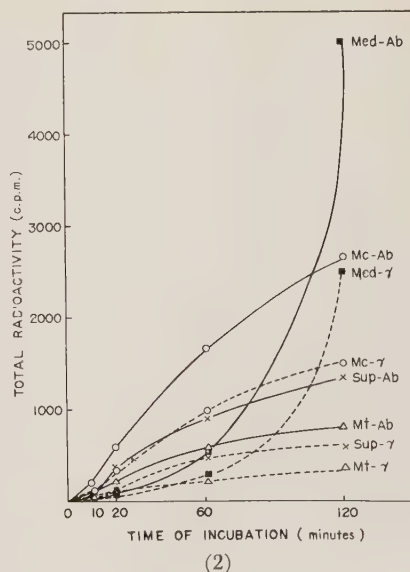


FIG. 2. Relative value of the specific activity of total γ -globulin of each cellular fraction at given times after the injection of C^{14} -glycine or S^{35} -methionine into hyperimmunized rabbit.

Expressed as per cent of the specific activity of microsomal total γ -globulin. (1) (4) (5): C^{14} -glycine (20 μ c) was used, (2) (3): S^{35} -methionine (40 μ c) was used, (4): average of two experiments, (5): normal rabbit.



(1)



(2)

FIG. 3. Time course of the total radioactivity of antibody and other γ -globulin of each cellular fraction and medium during the incubation of spleen slices with C^{14} -glycine.

Each Warburg flask, containing 1 g. of spleen slices, 2.6 ml. of the modified Krebs-Ringer phosphate and 0.4 ml. of C^{14} -glycine (1.29 μ moles) was incubated at 37°C for given times under the gas phase of oxygen. The reaction was started after 10 minutes equilibration period. Mc-Ab, Mt-Ab, Nuc-Ab, Sup-Ab, Med-Ab: antibody of microsomes, mitochondria, nuclei, supernatant and medium respectively; Mc- γ , Mt- γ , Nuc- γ , Sup- γ , Med- γ : other γ -globulin of each cellular fraction.

The Incorporation of C^{14} -Glycine into Antibody and Other γ -Globulin of Each Cellular Component by Hyperimmunized Spleen Slices—After the incubation of hyperimmunized rabbit spleen slices with C^{14} -glycine, antibody and other γ -globulin were separated from each cellular fraction by the immunological methods described in "Experimental" and the total radioactivity of each protein fraction was determined. Two typical time courses are shown in Fig. 3 (1) (2). Radioactivity appeared most rapidly in the antibody of microsomal fraction, whereas the labeling of that of other cellular fractions showed a delay of about 10 minutes and that in the medium had a longer delay of about 20 minutes, and then increased rapidly with time up to 2 hours. The same was observed with regard to the labeling of other γ -globulin of each cellular component, although the extent of the labeling was lower than that of the antibody of

the corresponding cellular fraction.*

It was also found that the total radioactivity of antibody or other γ -globulin of the microsomes is remarkably higher than that of the other cellular fractions.

The time relationship of the specific activity of the total γ -globulin including antibody of each cellular fraction was shown in Fig. 4.

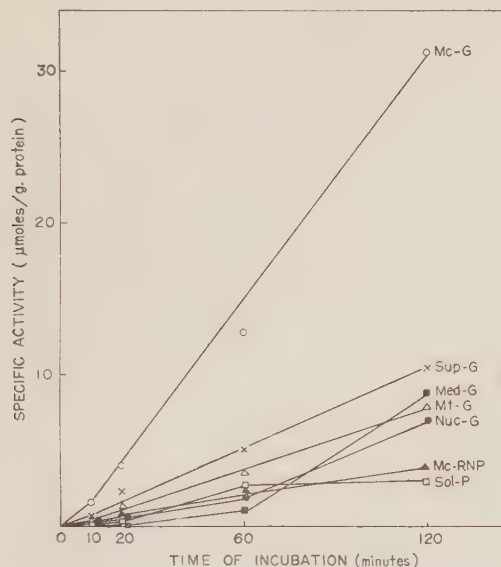


FIG. 4. Time course of the specific activity of the total γ -globulin of each cellular components, microsomal ribonucleoprotein and total soluble protein.

The experimental conditions were the same as in Fig. 3. Mc-G, Mt-G, Nuc-G, Sup-G, Med-G: total γ -globulin of microsomes, mitochondria, nuclei, supernatant and medium; Mc-RNP: microsomal ribonucleoprotein; Sol-P: total soluble protein.

The similar time course was obtained as in the former experiment. It was noticeable that no appreciable delay was observed in the labeling of microsomal γ -globulin and that the specific activity of the microsomal total γ -globulin was also remarkably higher than that of the other cellular fractions during the course of incubation, while no ap-

* The total radioactivity of the unspecific protein (see Experimental) of microsomes was about 1 per cent of that of the microsomal total γ -globulin.

preciable difference was observed among the specific activity of the total γ -globulin of mitochondria, nuclei and supernatant fractions, as also shown in Fig. 5. The specific activity of the total γ -globulin in medium was lower than that of each cellular component up to 1 hour of incubation. It was of interest that the specific activity of the total γ -globulin, especially that of microsomes, was remarkably higher than not only that of total soluble protein but also that of microsomal ribonucleoprotein even at a short incubation period.

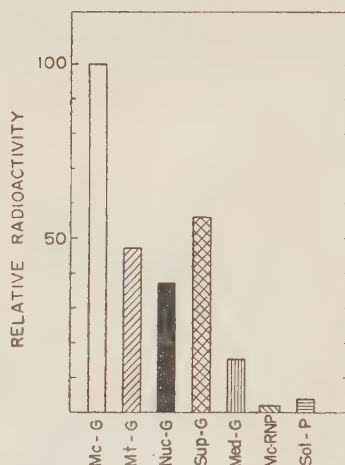


FIG. 5. Relative value of the specific activity of the total γ -globulin of each cellular component and medium after 60 minutes incubation of spleen slices.

Expressed as per cent of the specific activity of microsomal total γ -globulin. Average value of 6 experiments. The experimental conditions were the same as in Fig. 3. Mc-G, Mt-G, Nuc-G, Sup-G, Med-G: Mc-RNP; Sol-P: the same as in Fig. 4.

In order to obtain more definite evidence that the microsomal total γ -globulin was the precursor of that of other cellular fractions, the time course of the specific activity of the total γ -globulin of each cellular fraction was examined during the incubation of labeled spleen slices in the medium containing no radioactivity. The labeled slices were prepared by the preincubation of the hyperimmunized spleen slices with C^{14} -glycine, followed by

washing with non-labeled medium. As shown in Fig. 5, the specific activity of the total γ -globulin of microsomes decrease from almost the beginning of incubation in unlabeled medium, whereas that of the total γ -globulin of other cellular fractions increased for about 30 minutes and then decreased, and that in medium increased continuously from the beginning of incubation.

These results, in good agreement of those of our *in vivo* experiments, may indicate that the hyperimmunized spleen slice has a high activity in the formation of labeled γ -globulin, especially in that of antibody, and that the microsome is the main site of the synthesis of antibody and other γ -globulin.

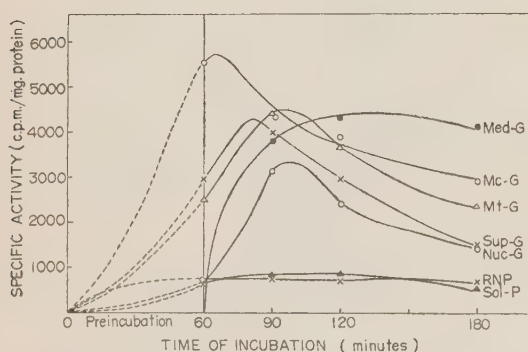


FIG. 6. Release of total γ -globulin from each cellular fraction of pre-labeled spleen slices.

After preincubation of spleen slices with C^{14} -glycine at 30°C for 60 minutes under the same conditions as in Fig. 3, the slices were washed with Krebs-Ringer phosphate solution and incubated in non-labeled medium for the periods indicated.

The Incorporation of C^{14} -Amino Acid into Antibody and γ -Globulin by a Cell Free System— From the results of the experiments described above, it seemed reasonable to investigate whether a cell free system consisting of microsomes and the pH 5 fraction (37) had activity to incorporate C^{14} -amino acid into antibody and other γ -globulin, since there were many indications that this system might provide the main pathway for the protein biosynthesis in microsomes. At first investigation was made of the incorporation of C^{14} -leucine into

the microsomal total γ -globulin without separation of antibody. Since it was found that the rat liver pH 5 fraction could replace the spleen pH 5 fraction in the incorporation into both microsomal ribonucleoprotein and the total γ -globulin and was found to have higher activity in some experiments, the pH 5 fraction from rat liver was used in the following experiment.

The components required for the incorporation in cell free system was shown in Table I. In the absence of the pH 5 fraction, there was a low level of incorporating activity, and this reduction was remarkably when once washed microsomes were employed. It was of interest that GTP was indispensable for the incorporation, together with ATP and an ATP generating system. The results were in good agreement with those of Keller and Zamecnik (28), concerning the incorporation of C^{14} -amino acids into the total microsomal proteins by the liver cell free system, and suggested the participation of the soluble RNA in the incorporation as first show by Hoagland *et al.* (31). The preincubation of the reaction mixture with a low concentration of RNase (0.25 μg . per ml.) remarkably reduced the incorporation. Also the preincubation of the pH 5 fraction with RNase followed by reprecipitation at pH 5 inhibited the incorporation, as compared with that of the control experiment, in which the preincubation of the pH 5 fraction without RNase followed by reprecipitation at pH 5 was employed. The results may indicate the participation of the soluble RNA in the incorporation, although the decrease in the amino acid activation by this procedure must be considered, which was reported by the present author (34) and Von der Decken and Hultin (35). As also shown in this Table, the preincubation of the pH 5 fraction at 30°C for 30 minutes (without RNase) followed by reprecipitation alone was found to reduce the incorporation as compared with that by the original pH 5 fraction. The result may indicate the importance of the end group of the soluble RNA in the process of the incorporation, since it was

TABLE I
Incorporation of C^{14} -Leucine into Total γ -Globulin by Cell Free System

	Specific activity (counts/min./mg.)	
	γ -Globulin	Ribonucleoprotein
(A) Complete system	610	117
Minus ATP	85	15
Minus GTP	85	37
(B) Complete system	1,455	350
Minus pH 5 fraction	690	225
(C) Complete system (washed microsomes)	2,990	395
Minus pH 5 fraction	185	83
(D) Complete system	3,960	635
Plus RNase	641	99
(E) Complete system	6,106	1,200
Preincubated pH 5 fraction with RNase ¹⁾	118	49
Preincubated pH 5 fraction without RNase ²⁾	2,520	312

(A) The complete system; 5 mM $MgCl_2$, 50 mM KCl , 10 mM $KHCO_3$, 50 mM Tris buffer (pH 7.9), 1 mM ATP, 0.25 mM GTP, 43 m μ C^{14} -leucine (total counts, 6.2×10^5 counts/min.), 20 mM phosphocreatine, 100 μ g. of creatine kinase, about 8 mg. protein of spleen microsomes and about 6 mg. protein of rat liver pH 5 fraction, in a total volume of 2 ml. Incubated at 30°C for 30 minutes.

(B) The same conditions as in (A).

(C) The same conditions as in (A), except that once washed microsomes were used.

(D) The incubation mixture with C^{14} -leucine was preincubated with or without 0.25 μ g. RNase per ml. for 10 minutes at 30°C.

(E) The pH 5 fraction was preincubated with¹⁾ or without²⁾ RNase (5 μ g./ml.) at 30°C for 10 minutes, and then reprecipitated at pH 4.7 (34).

shown by Hecht and others (36) that such treatment caused the loss of the end group of the soluble RNA. It was also observed that there was a good parallelism between the inhibition of the labeling of the total γ -globulin and that of the ribonucleoprotein, indicative of the importance of the ribonucleoprotein in the biosynthesis of γ -globulin in microsomes.

To obtain the more direct evidence for the participation of the soluble RNA in the γ -globulin synthesis, the transfer of C^{14} -amino acids from the soluble RNA to the total γ -globulin was investigated, employing soluble RNA labeled with hydrolysate of C^{14} -algal protein.

The time course of the reaction (Fig. 7)

showed the more rapid labeling of the microsomal total γ -globulin, as compared with that in the case of the labeling with free C^{14} -amino acid shown in Fig. 8. It was further observed that the total radioactivity of the unspecific protein was very low, indicating the formation of true labeled γ -globulin in this case.

The requirement for this transfer was then examined (Table II). The pH 5 supernatant fraction from hyperimmunized rabbit spleen was necessary for the incorporation, although it could be replaced by the rat liver pH 5 supernatant fraction. Employing an enzyme fraction partially purified by ammonium sulphate fractionation from rat liver pH 5 supernatant, it was demonstrated that not only ATP but also GTP were required

TABLE II

The Labeling of Microsomal Total γ -Globulin by C^{14} -Amino Acids Bound to S-RNA

(1)

	Total radioactivity (counts/min.)	
	γ -Globulin	Ribonucleoprotein
Complete system	104	1,579
Plus rabbit spleen pH 5 supernatant ¹⁾	129	1,027
Minus pH 5 supernatant	28	316

(2)

	Total radioactivity (counts/min.)	
	γ -Globulin	Ribonucleoprotein
Complete system	332(39 ³⁾)	650
Minus ATP	33(10 ³⁾)	126
Minus GTP	134(12 ³⁾)	290
Minus GTP, ATP, ATP generating system	21(3 ³⁾)	120
Plus rat liver pH 5 supernatant ²⁾	404(55 ³⁾)	778

(1) The complete system was same as in Fig. 7, except that the soluble RNA (87 μ g., 20,000 counts per minute) labeled with an acid hydrolysate of algal C^{14} -protein was used. The incubation was carried out at 30°C for 30 minutes.

1) Rat liver pH 5 supernatant fraction was replaced by rabbit spleen pH 5 supernatant fraction.

(2) The complete system was the same as in (1), except that pH 5 supernatant fraction was replaced by an enzyme fraction (6 mg. of protein) described in the text and the soluble RNA (58 μ g., 28,700 counts per minute) was used. The incubation was carried out at 30°C for 15 minutes.

2) The enzyme fraction was replaced by the pH 5 supernatant fraction.

3) Total radioactivity of unspecific proteins.

TABLE III

Incorporation of C^{14} -Leucine into Microsomal Antibody and Other γ -Globulin by Cell Free System

Total radioactivity (counts/min.)			
Antibody	Other γ -globulin	Unspecific protein	Ribonucleoprotein
53	175	—	2,079
70	172	15	1,643

The experimental conditions were the same as in Table I (A). After removing unspecific protein, the antibody and other γ -globulin were precipitated by adding antigen and antiserum respectively as described in the text.

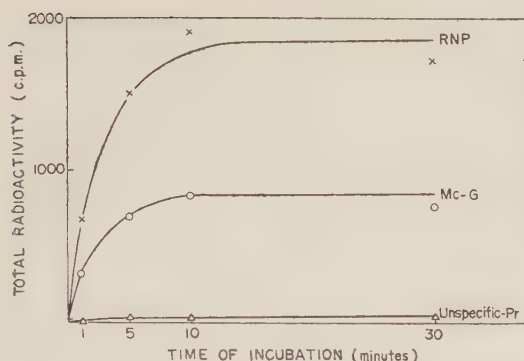


FIG. 7. Time course of the labeling of microsomal total γ -globulin and ribonucleoprotein by C^{14} -amino acids bound to soluble RNA.

The spleen microsomes once washed with the suspending medium (8 mg. of protein) and pH 5 supernatant fraction (7 mg. of protein) were incubated at 30°C for given time with the labeled soluble RNA (58 μ g., 28,700 counts per minute) in the medium, containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM $KHCO_3$, 50 mM Tris buffer (pH 7.9), 1 mM ATP, 0.25 mM GTP, 20 mM phosphocreatine, 100 μ g. of creatine kinase and 10 mM glutathione in a total volume of 2 ml. RNP: microsomal ribonucleoprotein, Mc-G: microsomal total γ -globulin, Unspecific-Pr: unspecific protein.

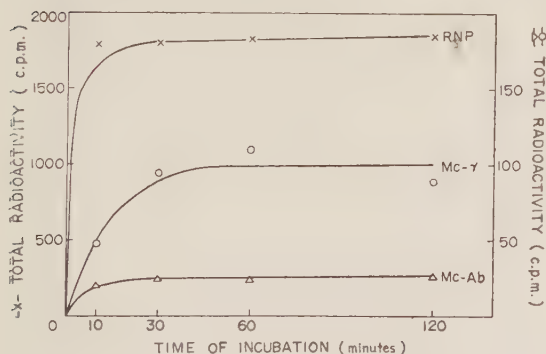


FIG. 8. Time course of the labeling of the microsomal antibody and γ -globulin by cell free system.

The experimental conditions and analytical methods were the same as in Table III. RNP: microsomal ribonucleoprotein, Mc- γ : microsomal γ -globulin, Mc-Ab: microsomal antibody.

for the incorporation into both microsomal total γ -globulin and ribonucleoprotein.

In order to determine to what extent the

labeling of the total γ -globulin was due to that of antibody, the total radioactivity of the microsomal antibody and other γ -globulin were determined after the incubation of hyperimmunized spleen microsomes with C^{14} -leucine in the presence of the rat liver pH 5 fraction, GTP, ATP, and an ATP generating system. Table III shows that the total radioactivity of antibody was lower than that of other γ -globulin, although it was higher than that of the unspecific protein. This result was somewhat different from that obtained in the slice experiment in which total radioactivity of antibody was higher than that of other γ -globulin. The time course of the reaction are shown in Fig. 8.

DISCUSSION

It must first be mentioned that identification of the γ -globulin fraction released from each cellular component was performed with immunological procedures. Owing to its high sensitivity, the results may suggest that our antibody and γ -globulin fraction represent nearly the same characteristics as serum antibody and γ -globulin respectively, although it is hoped to demonstrate unequivocally that isolated radioactive protein has all the characteristics of specific antibody or serum γ -globulin. The low level of radioactivity of unspecific protein obtained by the treatment with unrelated antigen and its antibody in both cases of slice or cell free system may be in favor of this view.

There have been many recent studies indicating that the microsome or its ribonucleoprotein particle (ribosomes) is the main site in protein synthesis in animal tissues (see the review by Hoagland (37)).

Recent works concerning the synthesis of plasma protein were in favor of this view (15-18). The results of the present experiment with immunized spleen *in vivo* and in slices also indicate that antibody and other γ -globulin may be synthesized most actively in microsomes (probably ribosomes) of spleen cells in the form bound to them and then released to other cytoplasmic phase. This assumption is based on the following observa-

tions: 1. the specific activity of the total γ -globulin including antibody of microsomes is remarkably higher than that of other cellular fractions at each incubation period in the slice experiment and at the early time after the injection of labeled amino acid in the experiments *in vivo*; 2. there is a negligible delay before radioactivity is detectable in the microsomal antibody and other γ -globulin of immunized spleen slices, whereas a short but definite delay is observed in labeling of those of other cellular fractions. The above mentioned assumption was further confirmed by the experiment observing the time course of the release of the labeled total γ -globulin from each cellular components of the prelabeled slices.

These findings are generally in good agreement with those of Peters (15) concerning the labeling of serum albumin by chick liver slices and *in vivo*.

Our results also suggest that newly formed antibody and other γ -globulin may pass through soluble phase before secretion, since a long delay was observed before these labeled proteins appeared in the medium. The results of the experiment on the release of the labeled slices is also in favor of such assumption. Similar results were reported by Peters (15) concerning the release of albumin into medium from liver slices and by Askonas *et al.* (38) concerning the release of antibody into medium from slices of spleen and lymph gland from the immunized rabbit.

The possibility was not completely ruled out that other cellular particulate component such as mitochondria or nuclei has some activity in synthesis of antibody and other γ -globulin. It must be mentioned that negligible incorporation into nuclear albumin was observed by Peters (15) in the case of liver slices. Therefore, the incorporation into nuclear antibody and other γ -globulin in our experiments suggests the possibility that the nuclei of immunized spleen cells perform the formation of these specific proteins. However it seems more likely that the nuclei or mitochondria may bind the newly formed antibody and other γ -globulin, and play a role as a

trap in their secretion, since the rate as well as the extent of the incorporation of C^{14} -glycine into these two specific proteins of the nuclei or mitochondria is not different from that of the supernatant fraction, from which postmicrosomal fraction was removed by precipitation at pH 4.8. Further experiments employing more precise fractionation of each cellular component to prevent the contamination of other fractions and those employing a cell free system with isolated mitochondria or nuclei are necessary before discussing such problems.

The incorporation of C^{14} -leucine into microsomal total γ -globulin was further shown in a cell free system from hyperimmunized spleen, consisting of the pH 5 fraction and microsomes. It must be emphasized that amino acid activating enzymes, soluble RNA and microsomes may provide the main pathway for the incorporation of labeled amino acid into γ -globulin, as presented by Zamecnik, Hoagland and their associates as the general mechanism for the incorporation into microsomal or ribosomal total proteins (37, 39). The following results are in favor of this view: 1. the pH 5 fraction is essential for the incorporation and the incorporation is very low with the pH 5 fraction preincubated with RNase followed by precipitation at pH 5. GTP is required for the incorporation together with ATP and an ATP generating system. This assumption was further confirmed by the finding that the transfer of C^{14} -amino acids actually occurred from the labeled soluble RNA to the microsomal total γ -globulin in the presence of the pH 5 supernatant fraction from liver or spleen, GTP, ATP and an ATP generating system. These results were consistent with the labeling of hemoglobin by reticulocytes cell free system which was reported by Schweet *et al.* (40, 41) and that of albumin by rat liver cell free system which was reported by the present authors (17, 18).

It must be noticed that the activity in formation of labeled antibody is greatly impaired in our cell free system. The further study seems necessary for elucidating whether

this is essentially due to the rupture of cellular structure or due to some other reasons.

SUMMARY

Employing hyperimmunized spleen, the incorporation of labeled amino acids into γ -globulin including antibody of each cellular component was investigated *in vivo* and in slices. The cell free system for the incorporation of C^{14} -amino acids into the microsomal γ -globulin was also studied. The following results were obtained.

1. The γ -globulin fraction of each cellular component obtained immunologically could be identified as serum γ -globulin by means of Orchterlony's gel diffusion method.

2. The specific activity of the total γ -globulin of microsomes was higher than that of other cellular fractions at an early hour (up to 1 hour) after the injection of C^{14} -glycine and S^{35} -methionine into hyperimmunized rabbit and became lower than that of mitochondrial fraction 2 hours after injection.

3. During the incubation of immunized spleen slices with C^{14} -glycine, the radioactivity appeared most rapidly in antibody and other γ -globulin of microsomes, whereas a short but definite delay (about 10 minutes) was observed in the labeling of these proteins of other cellular fractions, and a longer delay (about 20 minutes) in the labeling of these proteins in the medium. The total radioactivity of antibody of each cellular fraction was remarkably higher than that of other γ -globulin of the corresponding cellular fraction. It was further shown that the specific activity of the total γ -globulin of microsomes was remarkably higher than that of the other cellular fractions up to 2 hours and that in medium showed the lowest value up to 1 hour during the incubation of spleen slices with C^{14} -glycine.

4. During the incubation of labeled slices in non-labeled medium the specific activity of the microsomal total γ -globulin decreased from almost the beginning of the incubation and that of other cellular fraction increased up to 30 minutes and then decreased, and that in medium increased continuously up to

2 hours.

The results mentioned above may indicate that antibody and other γ -globulin are most actively synthesized in microsomes in the form bound to them, released into other cytoplasmic phase and then secreted into the medium.

5. The cell free system consisting of hyperimmunized rabbit spleen microsomes and the pH 5 fraction of rat liver incorporated C^{14} -leucine into the microsomal total γ -globulin. GTP was essential for the incorporation, together with ATP and an ATP generating system. Preincubation of the reaction medium with the low concentration of RNase as well as that of the pH 5 fraction with RNase followed by reprecipitation at pH 5 reduced the incorporation. The formation of labeled antibody was observed in this system, although the extent of labeling was very low.

6. The transfer of C^{14} -amino acids from soluble RNA into microsomal total γ -globulin was observed in the presence of the pH 5 supernatant fraction. The requirement for GTP, together with ATP and an ATP generating system, was shown with partially purified enzyme fraction from the rat liver pH 5 supernatant.

These results may indicate that amino acid activating enzymes, soluble RNA and microsomes provide the main pathway for γ -globulin synthesis in immunized spleen cells.

This work was supported by a Grant in Aid for Scientific Research from the Department of Education.

REFERENCES

- (1) Ogata, K., Ogata, M., Mochizuki, Y., and Nishiyama, T., *J. Biochem.*, **43**, 653 (1956)
- (2) Roberts, S., Adams, E., and White, A., *J. Biol. Chem.*, **174**, 379 (1948)
- (3) Ranney, H. M., and London, I. M., *Federation Proc.*, **10**, 562 (1951)
- (4) Keston, A. S., and Katchen, B., *J. Immunol.*, **76**, 253 (1956)
- (5) Askonas, B. A., and White, R. G., *Brit. J. Exp. Path.*, **37**, 61 (1956)

- (6) Omori, S., *Niigata Med. J.*, **74**, 91 (1960), *Acta Medica Niigata*, in press
- (7) Askonas, B. A., and Humphrey, J. H., *Biochem. J.*, **68**, 252 (1958)
- (8) Humphrey, J. H., and Sulitzeanu, B. D., *Biochem. J.*, **68**, 146 (1958)
- (9) Askonas, B. A., and Humphrey, J. H., *Biochem. J.*, **70**, 212 (1958)
- (10) Steiner, D. F., and Anker, H. S., *Proc. Natl. Acad. Sci., U.S.A.*, **42**, 580 (1956)
- (11) Stavitsky, A. B., and Wolf, B., *Biochim. et Biophys. Acta*, **27**, 4 (1958)
- (12) Wolf, B., and Stavitsky, A. B., *J. Immunol.*, **81**, 404 (1958)
- (13) La Via, M. F., Uriu, S. A., and Ferguson, L. A., *J. Immunol.*, **84**, 48 (1960)
- (14) Higashi, T., and Hasegawa, F., *J. Biochem.*, **48**, 71 (1960)
- (15) Peters, T. Jr., *J. Biol. Chem.*, **229**, 659 (1957), *J. Histochem. Cytochem.*, **7**, 224 (1959)
- (16) Campbell, P. N., Greengard, O., and Kernot, B. A., *Biochem. J.*, **74**, 107 (1960)
- (17) Ogata, K., Hirokawa, R., and Omori, S., *Biochim. et Biophys. Acta*, **40**, 178 (1960)
- (18) Hirokawa, R., *J. Japan. Biochem. Soc.*, **32**, 453 (1960)
- (19) Niklas, A., *Z. Physiol. Chem.*, **301**, 194 (1955)
- (20) Lardy, H. A., "Mannometric Techniques", Burgess Publishing Co., Minneapolis, 2nd ed., p. 288 (1957)
- (21) Kuby, S. A., Noda, L., and Lardy, H. A., *J. Biol. Chem.*, **209**, 191 (1954)
- (22) Keckwick, R. A., and Cannan, R. K., *Biochem. J.*, **30**, 227 (1936)
- (23) Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, **50**, 809 (1929), **58**, 137 (1933)
- (24) Hogeboom, G. H., Schneider, W. C., and Strie-
bich, M. J., *J. Biol. Chem.*, **196**, 111 (1952)
- (25) Campbell, P. N., *Biochem. J.*, **61**, 496 (1955)
- (26) Siekevitz, P., and Palade, G. E., *Federation Proc.*, **17**, 311 (1958)
- (27) Dixon, F. J., Maurer, P. H., Weigle, W. O., and Deichmiller, M. P., *J. Exp. Med.*, **103**, 425 (1956)
- (28) Keller, E. B., and Zamecnik, P. C., *J. Biol. Chem.*, **221**, 45 (1956)
- (29) Littlefield, J. W., and Keller, E. B., *J. Biol. Chem.*, **224**, 13 (1957)
- (30) Porath, J., and Flodin, F., *Nature*, **183**, 1057 (1959)
- (31) Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I., and Zamecnik, P. C., *J. Biol. Chem.*, **231**, 241 (1958)
- (32) Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1929)
- (33) Wilson, M. W., and Pringle, B. H., *J. Immunol.*, **73**, 232 (1954)
- (34) Ogata, K., Nohara, H., and Morita, T., *Biochim. et Biophys. Acta*, **26**, 656 (1957)
- (35) Von der Decken, A., and Hultin, T., *Exp. Cell Res.*, **17**, 188 (1959)
- (36) Hecht, L. I., Stephenson, M. L., and Zamecnik, P. C., *Proc. Natl. Acad. Sci., U.S.A.*, **45**, 505 (1959)
- (37) Hoagland, M. B., "The Nucleic Acids", Vol. III, Acad. Press Inc., New York, p. 349 (1960)
- (38) Askonas, B. A., Simkin, J. L., and Work, T. S., 'Proceedings of the 4th International Congress of Biochemistry', Vol. VIII, Pergamon Press, London, p. 181 (1960)
- (39) Zamecnik, P. C., *Harvey Lecture*, **54**, 256 (1960)
- (40) Schweet, R., Lamfrom, H., and Allen, E., *Proc. Natl. Acad. Sci., U.S.A.*, **44**, 1029 (1958)
- (41) Allen, E., and Schweet, R., *Biochim. et Biophys. Acta*, **39**, 185 (1960)

On the Determination of Serum Caeruloplasmin, and the Results of Its Measurement

By MITIYUKI SHIMIZU, YOSHIHIRO MARUYAMA, MASAO KUKITA,
YOICHI YANAGISAWA, TETSUO SATO⁺ and
SHIGEMASA OSAKI

(From Shimizu's Medical Clinic, Showa Medical School, Tokyo and the ⁺Department of Organic Chemistry, Tokyo Institute of Technology, Tokyo)

(Received for publication, March 16, 1961)

Caeruloplasmin is a copper-protein first isolated from porcine serum by Holmberg and Laurell (1, 2) in 1948. In their reports on the physiological action of caeruloplasmin, Holmberg and Laurell described a diamine-oxidase activity of caeruloplasmin by using dimethyl *p*-phenylenediamine as the substrate. Recently its activity as a hematopoietic factor was reported by Shimizu (3), one of the present authors. Caeruloplasmin increases in plasma during sudden anemia. It is also present in normal human blood and is thought to act as a controlling factor of hematopoietic system.

The reduced type of caeruloplasmin promotes increased cellular division of already existent erythrocytic precursors while its oxidized type accelerates the growth of the young cells of granulopoietic system (4). Caeruloplasmin markedly increases at the appearance of anemia in rabbits caused by bleeding, phenylhydrazine hemolysis (5), and hemolysis by fibrinolytic enzyme (6). Caeruloplasmin lowers serum copper level (7), increases catalase activity of rabbit liver, accelerates tissue respiration, accelerates absorption and storage of iron resulting in a marked increase of iron in liver (8), and increases the number of young cells of erythropoietic and granulopoietic systems, eventually to bring about hyperplastic pictures in bone marrow but it does not stimulate extramedullary hematopoiesis (9).

According to our collaborators Osaki and Kaya *et al.* (10, 11), caeruloplasmin isolated in crystalline state from porcine

serum was analyzed by one-dimensional paper chromatography and was found to be a copper-glycoprotein containing 2 per cent of sugar, consisting of hexoses (glucose and mannose) and pentose (xylose). Its molecular weight, calculated from S_{w20} and D_{w20} values is $162,000 \pm 1,700$.

It had been assumed that the hematopoietic factor in plasma was a glycoprotein or related substance (12-14) and now caeruloplasmin, which is a copper-glycoprotein, is also thought to be a hematopoietic factor.

In the present paper the method for determination of serum caeruloplasmin, devised by Rabin (15), estimating its diamine-oxidase activity, was modified by use of caeruloplasmin crystals isolated from hog as a standard. Caeruloplasmin values in various diseases were measured, together with serum copper level when anemia was present, and the values were examined from the point of hematopoietic factor.

METHOD

1) Determination of Caeruloplasmin Reagents:

(1) 0.5% *p*-Phenylenediamine solution:

A neutral solution obtained by dissolving *p*-phenylenediamine hydrochloride in water and correctly neutralized with sodium hydroxide.

(2) 0.1 M Acetate buffer (pH 6.0).

(3) 0.1% Sodium azide solution

(4) 3% Sodium chloride solution

Procedure: Into a test tube of 1.8 cm. in diameter and 10 cm. in length, 2 ml. of acetate buffer (pH 6.0) is pipetted, 0.1 ml. of serum accurately measured is added, and the mixture is allowed to stand at room temperature for 20 minutes. To this mixture, 1 ml.

of *p*-phenylenediamine solution is pipetted with mixing and the mixture is warmed in a bath of 37°C for 30 minutes. The test tube is taken out of the bath, 1 ml. of 0.1% sodium azide solution is added, followed by 6 ml. of 3% sodium chloride solution, and the total volume of the liquid is brought to 10.1 ml. A blind test is carried out with the same quantity of the same reagents, inhibiting oxidation by addition of sodium azide solution before adding *p*-phenylenediamine solution to serum, and treated in a same manner.

Measurement: Extinction of various test solutions are measured by the Beckman Spectrophotometer, using a filter of 530 mμ and the blind test solution at zero extinction. Concentration of caeruloplasmin in mg./dl. is read from the standard calibration curve.

Preparation of the Standard Calibration Curve: Caeruloplasmin, crystallized from porcine serum, is dissolved in distilled water and its extinction at 610mμ is read on the Beckman Spectrophotometer. The concentration of caeruloplasmin is calculated from the following equation and dilution series are prepared by dilution of this solution to contain 5–60 mg./dl. of caeruloplasmin. The above-described procedure is carried out with 0.1 ml. of each of these standard solutions in place of serum, the extinction is read on the spectrophotometer, and the standard curve is drawn.

Caeruloplasmin (g./dl.) = $\frac{Ex}{10100} \times 162000$

where Ex=Extinction of the test solution at 610mμ
10100=Extinction of 1 mole of crystalline caeruloplasmin solution at 610 mμ
162000=Molecular weight of caeruloplasmin

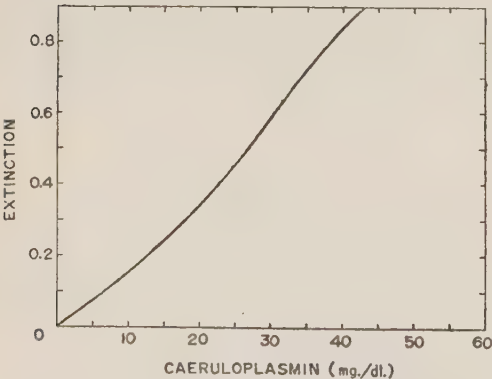


FIG. 1. Standard curve of caeruloplasmin. (Oxidase activity)

II) *Evaluation of the Amount of Copper in Caeruloplasmin*

Amount of copper (μg.) = $x \times \frac{8 \times 63.5}{162000}$

where 63.5=atomic weight of copper
8 =number of copper atoms contained in one molecule of caeruloplasmin
x =amount of caeruloplasmin (μg.)
III) *Determination of Serum Copper*—According to the Gubler's method (16).

EXPERIMENTAL RESULTS AND DISCUSSIONS

The serum caeruloplasmin value in normal healthy person was 12.6–15.9 mg./dl., with crystalline caeruloplasmin as the standard.

TABLE I
Serum Caeruloplasmin Value in Normal Healthy Persons

Name	Sex	Age	Serum Caeruloplasmin (mg./dl.)
T·Y	Male	29	14.4
K·I	//	30	14.5
M·S	//	43	15.4
K·K	//	43	15.9
N·K	//	28	14.8
N·M	//	29	13.8
S·W	//	25	12.6
T·B	Female	30	15.0
K·T	//	27	14.8
S·I	//	28	13.0
S·T	//	28	15.2
K·R	//	35	14.3
A·Y	//	50	13.9
S·A	//	33	15.0
K·S	//	10	14.8
K·Y	Male	15	15.3
M·R	//	23	13.8

Serum caeruloplasmin value was found to be high in infections, pregnancy, malignant tumor, and anemia, and low in Wilson's disease and chronic nephritis. Caeruloplasmin

TABLE II
Serum Caeruloplasmin Value in Various Diseases

Anemia					
Patient	Sex	Age	Diagnosis	Serum c.p. (mg./dl.)	C.R.P.
Y·I	Male	23	Hypoplastic anemia	23.4	
S·E	Female	53	//	24.2	
U·T	Male	25	Iron deficient anemia	23.0	
S·M	Male	30	Bleeding anemia	24.2	
A·O	Female	39	//	23.5	
S·O	Female	34	//	29.8	
T·K	Male	43	Normochromic anemia pulmo. tuberculosis	30.0	
M·S	Male	40	//	35.3	
N·T	Male	28	//	25.0	
Other Diseases·					
K·S	Female	30	Pyelitis	32.0	
H·N	Female	32	//	31.2	
T·T	Female	27	//	27.6	
K·T	Male	56	Pulmonary abscess	21.5	
A·K	Female	24	Rheumatoid arthritis	26.8	+++
S·O	Male	30	Endocarditis	27.6	+++
M·K	Male	21	//	28.8	
K·F	Male	49	Myocardial infarction	29.6	++
T·N	Female	30	Pleurisy	34.3	+++
A·U	Female	21	//	23.7	
G·O	Male	22	//	25.2	
S·K	Male	19	//	19.3	
A·F	Female	40	Dermatomyositis	43.9	+++
I·N	Male	25	Duodenal ulcer	15.1	
S·K	Male	32	//	15.0	
U·T	Female	25	//	16.3	
K·F	Male	30	Gastritis	16.3	
R·N	Male	55	Brainhemorrhage	15.0	
E·T	Male	35	Stomach ulcer	14.7	

TABLE II (continued)

Other Diseases					
Patient	Sex	Age	Diagnosis	Serum c. p. (mg./dl.)	C.R.P.
Y·S	Female	30	Gastritis	14.7	
S·H	Male	48	Diabetes mellitus	14.0	
E·S	Male	28	Stomach ulcer	13.5	
M·K	Female	45	Angina pectoris	11.6	
S·O	Female	38	Pneumonia	19.6	
N·E	Male	25	Hepatitis	15.5	
S·T	Male	32	//	15.4	
K·K	Female	28	//	15.9	
T·M	Male	49	Livercirrhosis	14.1	
Y·K	Female	50	Hypertension	17.1	
N·T	Female	49	//	18.0	
S·T	Male	53	//	15.9	
T·K	Male	45	//	15.8	
H·H	Male	59	//	16.0	
K·K	Male	53	//	14.2	
Y·S	Female	8	Wilson's disease	0	
G·M	Female	8	//	0	
K·T	Female	7	//	2.0	
Pregnancy					
Patient	Sex	Age	Month	Serum c. p. (mg./dl.)	C.R.P.
K·F	Female	21	4	34.4	
I·H	//	23	//	18.6	
A·S	//	28	//	19.2	
S·S	//	25	//	29.8	
M·S	//	24	5	14.8	
T·O	//	30	//	27.3	
A·Y	//	24	6	29.5	
N·A	//	28	//	24.1	
N·M	//	32	7	26.0	
S·N	//	24	8	23.9	

TABLE II (continued)

Pregnancy					
Patient	Sex	Age	Month	Serum c. p. (mg./dl.)	C.R.P.
O·Y	Female	29	9	28.5	
K·Y	//	32	//	34.5	
N·O	//	25	10	27.3	
H·N	//	22	//	27.6	
R·K	//	28	//	27.3	
S·Y	//	29	//	32.1	
Y·M	//	32	//	27.1	
N·W	//	24	//	29.1	
K·Y	//	25	//	33.1	
T·F	//	28	//	24.1	
Y·Y	//	23	//	35.1	
A·K	//	28	//	32.5	
Malignant Tumor					
Patient	Sex	Age	Organ	Serum Caeruloplasmin (mg./dl.)	C.R.P.
A·T	Male	47	Lung	33.3	##
M·N	Male	36	//	47.2	##
S·T	Female	45	Uterus	28.5	
N·K	//	53	//	30.1	
S·N	//	56	//	23.8	
M·S	//	47	//	16.8	
T·T	Female	37	Stomach	30.2	
H·K	Male	53	//	23.2	
S·K	//	42	//	23.8	
H·S	Female	49	//	20.5	
G·Y	Male	58	//	16.0	
A·I	//	55	Oesophagus	17.5	
M·O	//	66	//	18.3	

TABLE III
Serum Caeruloplasmin Value in Nephritis

Patient	Sex	Age	Serum Caeruloplasmin (mg./dl.)	Disease
K. F.	Male	15	17.2	Chronic nephritis
S. K.	Female	28	15.1	"
T. M.	"	18	6.7	"
T. F.	"	18	7.4	"
K. S.	"	24	16.3	"
M. S.	"	19	5.6	"
A. I.	"	20	6.5	"
F. T.	Male	18	6.0	"
T. N.	"	56	26.3	"
K. I.	"	19	15.7	"
S. N.	Female	42	18.0	"
S. I.	"	28	18.6	"
H. M.	Male	19	22.0	"
K. M.	Female	28	7.5	"
S. H.	"	22	26.3	" (Pregnancy VII M.)
M. S.	"	18	13.8	Nephrotic syndrome
T. H.	"	22	11.6	"
M. S.	Female	45	17.1	"
T. N.	Male	29	17.9	Acute nephritis
S. I.	"	14	17.8	"
K. K.	"	16	20.0	"
T. M.	"	16	22.4	"
Y. H.	"	18	20.2	"
O. S.	Female	20	29.1	"

value was generally high when C-reactive protein was positive.

In nephritic patients, caeruloplasmin value was high when the disease was accompanied by anemia or infections such as tonsillitis and pharyngitis, while the value was extremely in low tendency in chronic nephritis not accompanied with infection or

anemia.

Hematopoietic factor is thought to be formed chiefly in liver, but kidney is now being regarded as directly responsible for the production of hematopoietic factor (17, 18). Na et al (19) reported that nephrectomy in a dog decreased the activity of hematopoietic factor in plasma, from 15.2 per cent to 5.6 per

TABLE IV
Relation of Caeruloplasmin Copper to Serum Total Copper after Repeated Intramuscular Injection of Caeruloplasmin to Anemic Patients

No.	Name	Sex	Age	Day after injection	Serum copper ($\mu\text{g./dl.}$)	Serum caeruloplasmin (mg./dl.)	Copper content in caeruloplasmin ($\mu\text{g./dl.}$)	Ratio of caeruloplasmin copper to total copper (%)	Erythrocytes ($\times 10^4$)	Hemoglobin (by Sahli)	Reticulocytes (%)
1	Y. I.	Male	23	1st	172	11.5	36	20	329	54	5
				8th	167	19.6	61.4	36	315	51	10
				52nd	77	20.2	63.3	82	426	61	25
2	S. E.	Female	54	1st	180	20.3	64	35	360	63	11
				55th	151	22.9	72	47	349	59	13
				80th	100	18.2	57	57	408	67	14
3	G. H.	Male	43	1st	160	17.7	54	33	334	68	5
				22nd	127	31.3	98	77	345	64	7
				25th	125	29.0	91	73	377	71.5	10
				62nd	100	19.7	62	56	400	75	15
4	I. H.	Female	40	1st	192	17.2	54	28	268	32	44
				16th	86	18.5	58	67	309	36	51

cent during 24 hours but such findings were not observed on ligation of urethra. From these experiments, he concluded that the kidney either produced hematopoietic factor directly or retained a substance which liberated this factor at optional time into the blood when required.

In nephritic diseases, especially in chronic nephritis, caeruloplasmin was low, around 6 mg./dl., and hematopoietic factor is expected to decrease during kidney trouble. This agrees with the foregoing experimental result. However, in two cases of nephritic anemia with a high nitrogen in the blood of 71 mg./dl. and hypoplastic pictures of the bone marrow, caeruloplasmin value was high. This may be interpreted as the increase of hematopoietic factor accompanying nephritic anemia.

In the plasma, free copper is bound lightly with albumin and the amount is about 5 per cent of serum copper. The remaining 95 per cent is said to be copper in caeruloplasmin. In the present series of experiments, this ratio was not necessarily constant. In the case of anemia, free copper was present in larger amount than caeruloplasmin copper, and the ratio was not the same as in normal persons. This fact suggests that there might be an interference in the synthetic process of caeruloplasmin from copper.

The present authors demonstrated earlier that caeruloplasmin possessed the action of lowering serum copper level by repeated intramuscular injection of 2-4 mg. of reduced caeruloplasmin in the cases of anemia with high levels of serum copper and showed that the number of erythrocytes and reticulocytes increased with lowering of serum copper level. It was considered that the administered caeruloplasmin acted as a catalyst, resulting in a incorporation of copper into caeruloplasmin, and this caused an increase in serum caeruloplasmin value and lowering of serum copper level, the ratio of the two values becoming normal, and thus anemia tended to become better.

For example, in the case of No. 1 of Table IV of hypoplastic anemia, the values

during anemia were 172 μ g./dl. of serum copper and 36 μ g./dl. of caeruloplasmin copper, the rate of caeruloplasmin copper to total serum copper being 20 per cent.

With improvement of anemia, serum copper became 77 μ g./dl. and caeruloplasmin copper to total serum copper being 82 per cent.

In the case of No. 3 of normochromic anemia accompanying pulmonary tuberculosis, the values during anemia were 160 μ g./dl. of serum copper, the rate of caeruloplasmin copper to total serum copper being 33 per cent. With improvement in anemia, the values became 125 μ g./dl. of serum copper and 91 μ g./dl. of caeruloplasmin copper, which was 73 per cent of total serum copper.

These data suggest that administration of caeruloplasmin stimulates the incorporation of free copper into caeruloplasmin.

SUMMARY

Serum caeruloplasmin value was determined by use of a calibration curve prepared with crystalline caeruloplasmin in healthy persons as well as in various diseases. Results obtained were as follows:

1. The serum caeruloplasmin level in normal healthy persons was 12.6-15.9 mg./dl.
2. Serum caeruloplasmin level showed a high value in infections, pregnancy, anemia, and malignant tumor, and a low value in Wilson's disease and chronic nephritis.
3. Caeruloplasmin value was high in general when C-reactive protein was positive.
4. In anemia, caeruloplasmin copper is lower than serum copper value but the latter was lowered by administration of caeruloplasmin, the ratio of caeruloplasmin copper to total serum copper becoming normal, and there was improvement in anemia.

The authors wish to express their deep gratitude to Prof. K. Kodama for his kind guidance throughout this work.

REFERENCES

- (1) Holmberg, C. G., and Laurell, C. B., *Acta Chem. Scand.*, **1**, 944 (1947)
- (2) Holmberg, C. G., and Laurell, C. B., *Acta Chem. Scand.*, **2**, 550 (1948)

- (3) Shimizu, M., 'Symposium on 8th International Hematological Meeting', Tokyo, September, (1960)
- (4) Kato, K., and Shimizu, M., *Showa Med. Journal*, **20**, 1666 (1961)
- (5) Kuwatsuru, Y., and Shimizu, M., *Showa Med. Journal*, in the press.
- (6) Yagami, T., and Shimizu, M., *Showa Med. Journal*, **20**, 1264 (1960)
- (7) Ikeda, K., *J. Japan. Soc. Int. Med.*, **49**, 842 (1960)
- (8) Takahashi, A., and Shimizu, M., *Showa Med. Journal*, **20**, 1450 (1961)
- (9) Mitsuoka, S., and Shimizu, M., *Showa Med. Journal*, in the press.
- (10) Osaki, S., *J. Biochem.*, **48**, 190 (1960)
- (11) Osaki, S., Kaya, T., Kanazawa, T., Ogiwara, S., Sato, T., and Shimizu, M., *Proc. Japan. Acad.*, **37**, 54 (1961)
- (12) Rambach, W. A., Alt, H. L., and Cooper, J. A. D., *Blood*, **12**, 1101 (1957)
- (13) Rambach, W. A., Cooper, J. A. D., and Alt, H. L., *Proc. Soc. Exptl. Biol. Med.*, **98**, 602 (1958)
- (14) Slaunwhite, W. R., Mirand, E. A., and Prentice, T. C., *Proc. Soc. Exptl. Biol. Med.*, **96**, 616 (1957)
- (15) Ravin, H. A., *Lancet*, 726 (1956)
- (16) Gubler, C. G., Lahey, M. E., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M., *J. Biol. Chem.*, **196**, 209 (1952)
- (17) Jacobson, L. O., Goldwasser, E., Fried, W., and Plzak, L., *Nature*, **179**, 633 (1957)
- (18) Goldwasser, E., Fried, W., and Jacobson, L. O., *J. Lab. & Clin. Med.*, **52**, 375 (1958)
- (19) Naets, J. P., 'Symposium on 8th International Hematological Meeting', Tokyo, September (1960)

Some Chemical and Immunological Analyses of Proteins of Rat Ascites Hepatoma*

By HIDEMATUS HIRAI, KOYA SEKINE, AKIKO IJIMA and KOTARO WARABIOKA

(From the Department of Biochemistry, Faculty of Medicine, University of Tokyo, Tokyo, and The Cancer Research Institute, Tokyo)

(Received for publication March 18, 1961)

Many investigations have been made so far to discover any specific compounds which are present only in tumor cells but not in normal cells. Among the compounds some attention has been paid to the protein constituents in the tumor cells. In this type of investigation the immunological technique is one of the particularly helpful expedients because of its sensitivity for proving the specificity of the proteins in question although immunochemical study has not always been done successfully. Two main factors might be responsible for the failures, *i.e.*, tissue antigens are generally poor inducers of antibody, and the number of tissue antigens is so large as to defy analysis techniques with classic immunochemical methods.

The development of new immunological methods has led us to reevaluate the possibility of obtaining meaningful data in a field that has long been plagued by technical difficulties. Among these new methods are the immunization procedure developed by Freund (1) and refined precipitin reaction techniques, *i.e.*, the agar gel-diffusion method developed by Ouchterlony (2) and the immunoelectrophoresis contrived by Grabar (3).

Certain reports dealing with discovery of specific antigens in tumor cells have appeared. For example, Korngold (4) has shown the presence of specific antigens in several human tumors grown in rats or hamsters with aid of Ouchterlony technique. Korosteleva (5) has also demonstrated a specific

antigen in a mouse hepatoma by the precipitin method using extracted soluble proteins.

In the present study the authors report some chemical and immunochemical investigations of the proteins from rat ascites hepatoma cells (AH 49) and from livers. The advantage of using the ascites hepatoma cells for biochemical investigation is that: 1) the cells may be thoroughly washed free from other constituents of the rat, 2) the cells originate from a liver tumor induced by feeding rats with dimethyl-aminoazo benzene, hence the liver can be taken as a normal tissue for comparison with the tumor. In our experiments the soluble proteins were extracted at two different pH (4.8 and 7.0) and analyzed for their nucleic acid contents, electrophoretic and ultracentrifugal properties, and immunological specificity. Some specific proteins were demonstrated to be found in the tumor cells but neither in the liver nor in the blood serum of the rat.

EXPERIMENTALS

Materials—Rat ascites hepatoma, AH49, Sasaki Institute, Tokyo was used in this experiment. The ascites cells were repeatedly washed with a large volume of saline by slow centrifugation (about 30–50×g) and completely freed from contaminating red cells and others. Microscopic examination indicated complete purity of the cancer cells. Approximately 2–5 ml. of the cancer cells usually could be collected by the final centrifugation at 3,000 r.p.m. for ten minutes from ascitic fluid of a rat weighing 100–150 g.

As a comparison the livers of the normal rats were used. The livers were perfused extensively and treated in the same way as the packed cancer cells obtained by the above final centrifugation.

Methods—Nitrogen content of the preparation was

* The following abbreviations are used: trichloroacetic acid, TCA; ribonucleic acid, RNA; deoxyribonucleic acid, DNA; and optical density, O. D.

determined by the micro-Kjeldahl method. In the present paper the protein nitrogen is defined as nitrogen of the materials precipitated by 10% trichloroacetic acid (TCA). The Biuret reaction was also sometimes used for protein determination.

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) contents were determined by Bial's orcinol reaction (6) and Dische's diphenylamine method (7), respectively.

Electrophoretic and ultracentrifugal analysis were made by using the instruments of Hitachi Ltd. Co., Japan, Model HT-B and Model UCA-1 respectively.

Immunochemical analysis was carried out by the precipitin reaction with rabbit antisera using the immunoelectrophoresis technique developed by Grabar (3) and the agar diffusion method designed by Ouchterlony (2).

RESULTS

1. Chemical and Physicochemical Analyses

Extraction of the Proteins from Cancer Cells and Livers—One vol. of the tissue (cancer cells packed by centrifugation at 3,000 r.p.m. for 10 minutes, or extensively perfused livers) was homogenized with 3 vol. distilled water with Potter-Elvehjem homogenizer and then frozen and thawed three times. The pH of the homogenates was adjusted to 4.8 by addition of a small amount of *N* HCl followed by addition of 1 vol. of 0.5 *M* acetate buffer of pH 4.8. The concentration of salts added (acetate or phosphate) was always maintained at a constant level, *i.e.*, 0.1 *M*, and the temperature for extraction was kept at 1–2°C throughout the experiment. The homogenate thus obtained (H_1) was then centrifuged at 7,000 r.p.m. for 20 minutes and a slightly yellow (cancer cells) or yellow brownish (liver) but completely clear supernatant was obtained. The precipitate was washed with 1 vol. of 0.1 *M* acetate buffer and the wash fluids were combined with the supernatant giving $S_1(L_1)$ for liver) solution. $S_1(L_1)$ was saturated with ammonium sulfate and the precipitate formed was dissolved in about 1/3 vol. of water and dialyzed against vigorously stirred distilled water for several hours and then against a large volume of saline overnight. After completion of the dialysis the solution was centrifuged to

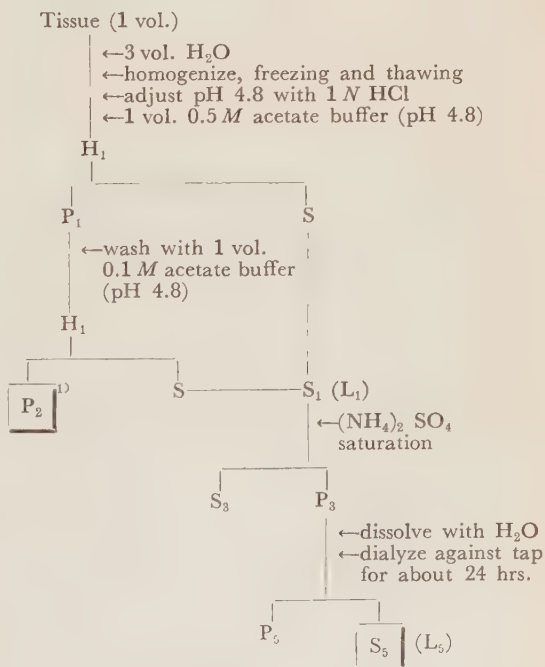
remove a small amount of insoluble material formed during the dialysis. The protein concentration of the final product named S_5 (L_5) was usually about 1–2 per cent.

After removal of $S_1(L_1)$ from the homogenate the remaining cell debris (P_2) were suspended in 1 vol. of water and the pH of the suspension was adjusted to 7.0 by the aid of a small amount of *N* NaOH followed by the addition of 3 vol. 0.17 *M* phosphate buffer of pH 7.0. The suspension (H_2) thus obtained was again frozen and thawed three times followed by centrifugation at 15,000 r.p.m. for 30 minutes. A slightly turbid and yellowish supernatant was decanted and the precipitate was washed with 1 vol. of the phosphate buffer. The wash solution was combined with the supernatant giving $S_2(L_2)$

SCHEME I

Fractionation of ascites cell or liver proteins

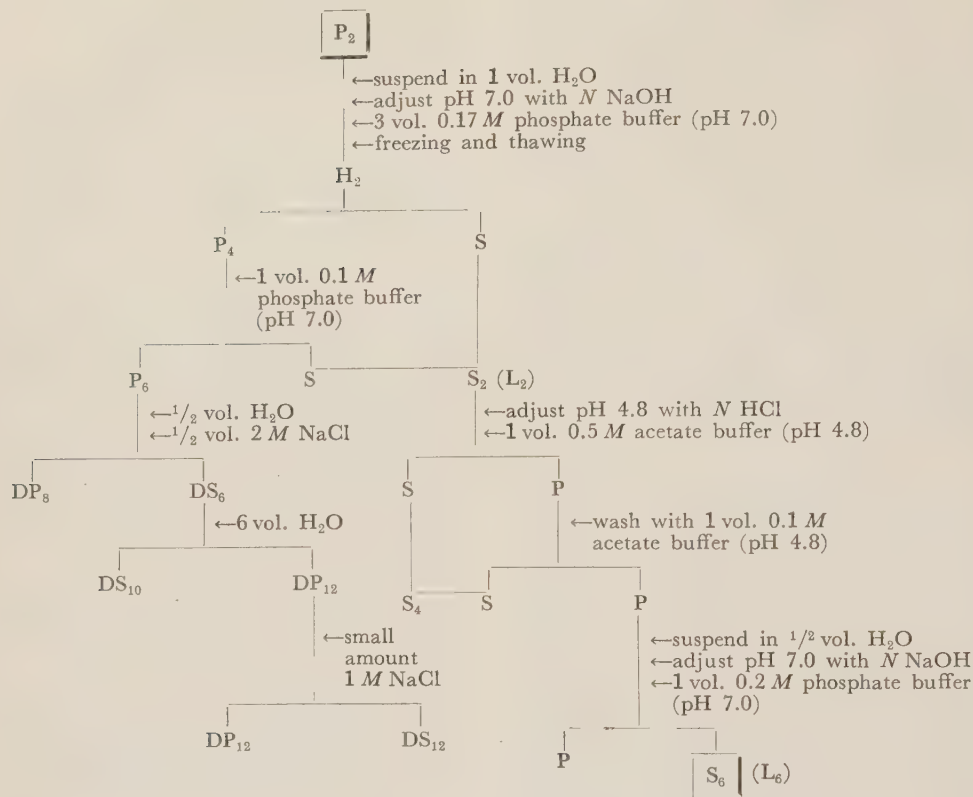
A



¹⁾ See the next page.

solution. The pH of S_2 was adjusted 4.8 with the aid of *N* HCl and 1 vol. of 0.1 *M* acetate buffer. A large amount of

B



white precipitate was formed and the supernatant was practically protein free. The precipitate was washed with 1 vol. of 0.1 *M* acetate buffer of pH 4.8 and then redissolved in pH 7.0 phosphate buffer with the aid of addition of *N* NaOH.

A final extract named S₆(L₆) which looked slightly turbid and yellow was thus obtained. The protein concentration of S₆(L₆) was usually 1.5–2.5 per cent. The scheme of the extraction above described is shown in Scheme 1-A and B.

Contents of the Fractions in Ascites Cells and Livers—A portion of each fraction shown in Scheme I was taken out and the total and non-protein nitrogen were determined. The results shown in Table I represent the content of protein nitrogen (10% TCA precipitable nitrogen) in each fraction as per cent of total nitrogen of the cells or tissue. Total nitrogen of ascites cells consists of 87.8%

TCA precipitable nitrogen and 12.2% non-protein nitrogen, and that of the livers consists of 85.3% protein nitrogen and 14.7% non-protein nitrogen.

The percentage of the total proteins extractable at pH 4.8 is obviously higher in livers (L₁, 17.7%) than in the ascites cells (S₁, 12.5%), on the other hand the portion of total protein extracted at pH 7.0 higher in ascites cells (S₂, 19.4%) than in livers (L₂, 13.9%). These results indicate that the cancer cells contain much more nucleoproteins than do liver cells as shown later.

The yield of the final fractions (S₅, L₅, S₆, L₆) was quite variable but approximately 50–70% of the theoretical value calculated from the amount of S₁(L₁) or S₂(L₂).

Optical Properties of the Fractions—As shown in Table II the O.D. values of S₅ solution of 1 mg. N/ml. concentration are higher than those of L₅, however the ratio

of E_{260} to E_{278} are about the same. O.D.s of S_6 or L_6 are several times higher than those of S_5 or L_5 . The O.D.s of L_6 are distinctly higher than those of S_6 although the ratios of E_{260} to E_{278} again are about the same.

As shown in Fig. 1 the absorption spectrum of S_5 coincides well with that of human serum albumin but that of S_6 indicates a good agreement with the spectrum of DNA or RNA isolated from yeast. The spectra of liver fractions indicated essentially

TABLE I
Content of Fractions in Ascites Cell and Liver

Sample	Number of samples	Fraction			
		H ₁	H ₂	S ₁ (L ₁)	S ₂ (L ₂)
Ascites cells	5-7	87.8 (83-93)	67.3 (62.5-76)	12.5 (9.1-15.6)	19.4 (13.6-26.5)
Livers	3	85.3 (82-91)	64.3 (56-72)	17.7 (16-19.8)	13.9 (10.1-17.3)

The figures give the average content of protein in each fraction as per cent of total nitrogen of the tissues. The figures in the parenthesis show the range of the values.

TABLE II
Optical Property of S_5 (L₅) and S_6 (L₆) Fractions in Ascites Cell and Liver

Sample	Number of sample	S_5 (L ₅)			S_6 (L ₆)		
		E_{260}	E_{278}	E_{260}/E_{278}	E_{260}	E_{278}	E_{260}/E_{278}
Ascites cells	5	4.65 (4.0-5.3)	6.62 (6.2-7.7)	0.70 (0.64-0.77)	40.4 (35.2-46.0)	27.2 (23.5-32.0)	1.49 (1.42-1.55)
Livers	3	4.00 (3.7-4.7)	5.55 (4.6-6.6)	0.72 (0.68-0.79)	59.5 (48.5-69.0)	37.0 (32.0-40.0)	1.59 (1.51-1.70)

Conc. of solution: 1 mg. N/ml.; optical path: 10.0 mm. Figures in parenthesis show the range of the values.

TABLE III
Nucleic Acid Contents of Fractions in Ascites Cell and Liver

Sample and kind of nucleic acid	Number of sample	H ₁	H ₂	S ₂ (L ₂)	S ₅ (L ₅)	S ₆ (L ₆)
Ascites Cell RNA	5-6	0.86 (0.71-1.05)	0.94 (0.69-1.15)	1.32 (0.97-2.13)	trace	1.22 (0.71-1.83)
Ascites Cell DNA	5-6	0.79 (0.60-0.92)	0.87 (0.65-1.00)	0.66 (0.39-1.23)	trace	0.83 (0.57-1.03)
Liver RNA	3	0.63 (0.43-0.89)	0.70 (0.50-1.05)	1.36 (0.98-2.05)	trace	1.47 (1.14-1.80)
Liver DNA	2-3	0.32 (0.27-0.42)	0.30 (0.23-0.42)	0.47 (0.46-0.47)	trace	1.12 (0.83-1.70)

Unit: mg. nucleic acid/mg. protein nitrogen.

Figures in parenthesis show the range of the values.

the same results as those of the corresponding fractions of ascites cells. These spectra indicate that the $S_5(L_5)$ fractions seem to be

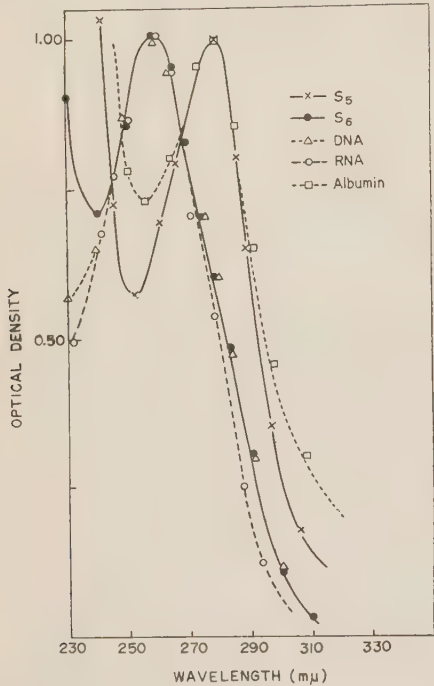


FIG. 1. Absorption spectra of ascites cell proteins.

of a pure protein nature while the $S_6(L_6)$ fractions are of nucleoprotein nature. The analytical data given in the next section will

In the Table it is observed that the nucleic acid contents of ascites cell homogenates are higher than in liver homogenates. This will be explained in that the nuclues of the ascites cells occupies a much larger space in the cell than does the nucleus in the liver cells.

Electrophoretic Analyses—Fig. 2-A shows ascending patterns of some cases of S_5 or L_5 analyzed by Tiselius' apparatus in pH 8.0 phosphate buffer. Although the mobility of the fastest peak is slightly larger than that of serum albumin, almost all peaks are approximately in the range of the mobilities of serum proteins. Each of the samples showed a quite reproducible pattern having about nine peaks. S_5 contains much more of the components of slower mobility than are found in L_5 but it is hard to recognize an essential difference between S_5 and L_5 .

Fig. 2-B shows the patterns of nucleoproteins, S_6 and L_6 . In those patterns we observe six or seven recognizable peaks and relatively higher amounts of the components of middle mobility. No essential difference is observed between S_6 and L_6 .

Ammonium Sulfate Fractionation of S_5 and S_6 —The samples were fractionated by successive addition of ammonium sulfate. The precipitates obtained successively at 25, 37 and 50% concentration of the salts for S_5 , 12.2, 35.7 and 50% for S_6 were dialyzed

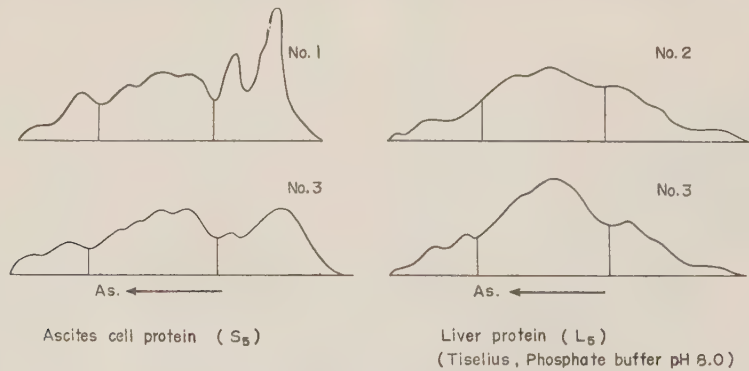
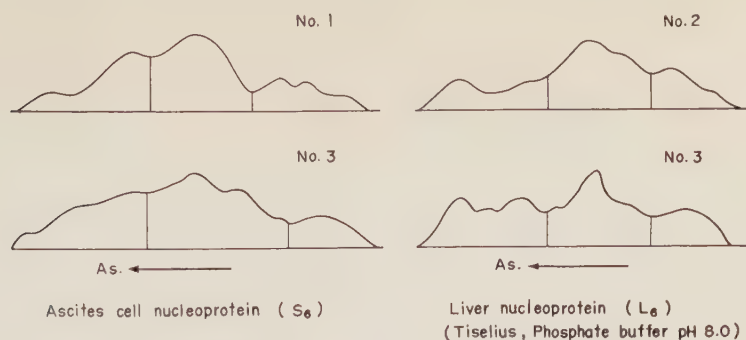
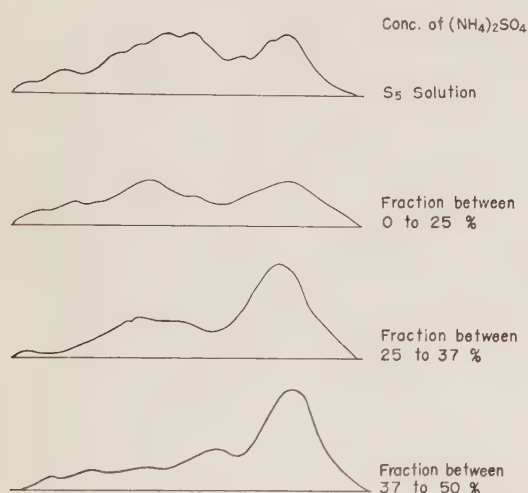
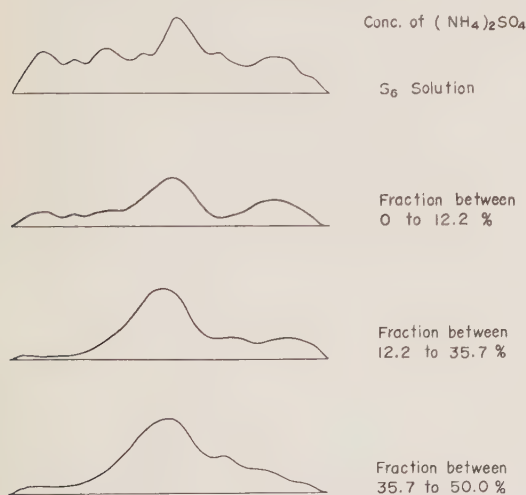


FIG. 2-A. Electrophoretic patterns of S_5 and L_5 .

present ample evidence for this assumption.

Nucleic Acid Contents of the Fractions—Table III represents mg. of RNA and DNA per mg. of protein nitrogen of the samples.

against water and the buffer for electrophoresis. These concentrations of the salt were chosen by analyzing the salting out curves of the samples.

FIG. 2-B. Electrophoretic patterns of nucleoproteins S_6 and L_6 .FIG. 2-C. Electrophoretic patterns of ascites cell proteins (S_5) fractionated with $(NH_4)_2SO_4$.FIG. 2-D. Electrophoretic patterns of ascites cell nucleoproteins (S_6) fractionated with $(NH_4)_2SO_4$.

As shown in Fig. 2-C each fraction still has many peaks but fractions obtained at higher concentrations of the salt contain a larger amount of components of slower mobility. In other words, higher concentration of the salt is required to precipitate of slow components. Fractionation of S_6 by ammonium sulfate was not satisfactory because it formed a lot of insoluble materials after the dialysis. The nucleoproteins seem readily to be denatured by the addition of the salt at high concentration. Fig. 2-D indicates the electrophoretic patterns of each successive fraction after the removal of insoluble materials. The fraction obtained by 50 per cent of the salt shows a relatively

TABLE IV

Sedimentation Constant (S_{20}) of Proteins of Ascites Cells, Liver and Serum

	Principal component	Faster component
Serum	4.0	7.3
Liver L_5	3.5	—
Liver L_6	3.3	—
Ascites S_5	4.5	6.1
Ascites S_6	4.5	10.8

large peak of middle mobility. The solubility of this fraction appears to be quite similar to that of serum albumin.

Ultracentrifugal Analysis—The proteins and their subfractions obtained by salting out with ammonium sulfate were subjected to ultracentrifugal analysis. Unexpectedly, generally all of these samples showed a main

component of sedimentation constant around 4 which is close to that of serum albumin. S_5 and S_6 showed a slight amount of more rapidly sedimentating components which were roughly close to serum globulin but L_5 and L_6 showed almost none of such rapid components.

Sedimentation constants of the samples are summarized in Table IV.

II. Immunochemical Analyses

Immunization of Rabbits—The antigenicity of the proteins extracted was very weak in

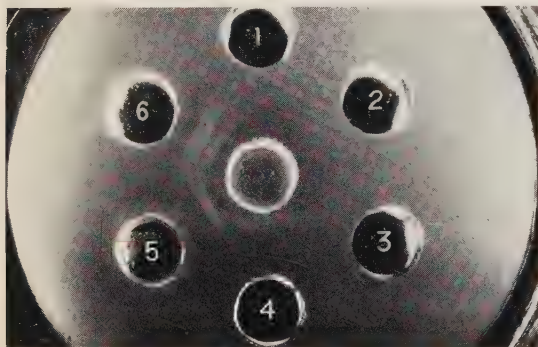


FIG. 3-A. Agar diffusion precipitin reaction.

Center Cup: Anti- S_5 serum

1: Saline 4: Serum

2: S_6 5: L_5

3: L_6 6: S_5



FIG. 3-C. Agar diffusion precipitin reaction.

Center Cup: Absorbed anti- S_5 serum

1: Saline 4: Serum

2: S_6 5: L_5

3: L_6 6: S_5

general. Solutions of S_5 or S_6 were extensively given intravenously to either rabbits or chickens but the precipitin titer obtained was very low. However, with Freund's

adjuvant, S_5 gave an antisera of a quite high titer which could be available for immunological analysis of the proteins extracted. Immunization was carried out as follows:

S_5 or S_6 was suspended in Freund's adjuvant at a protein concentration of 8 mg/ml., and 2 ml. of each suspension were given to each rabbit subcutaneously. After four weeks from the first injection the precipitin titer measured was 4 times dilution against S_5 and only two times against S_6 . In the fifth week 10 mg. of the S_5 or S_6 in

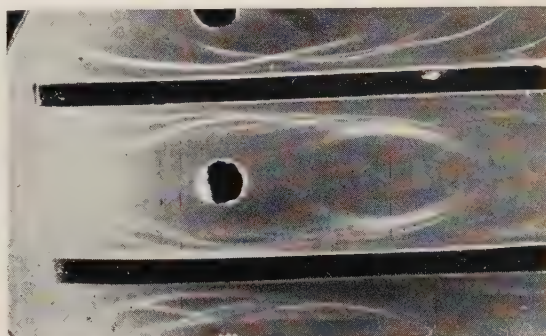


FIG. 3-B. Immunoelectrophoresis of S_5 , L_5 and rat serum with anti- S_5 serum.

Upper Cup: Rat serum

Middle Cup: S_5

Lower Cup: L_5

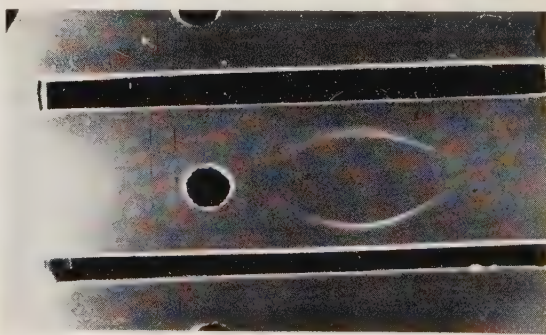


FIG. 3-D. Immunoelectrophoresis of S_5 , L_5 , and rat serum with absorbed anti- S_5 Serum.

Upper Cup: Rat serum

Middle Cup: S_5

Lower Cup: L_5

buffer solution were given intravenously and in the eighth week another 15 mg. of the antigen with Freund's adjuvant were injected subcutaneously. At the end of the

third month the rabbits were sacrificed. By that time the antibody titer was 32-64 times dilution against S_5 and 8-16 times dilution against S_6 . Antiserum from each rabbit was pooled and desensitized by heating at 56°C for 30 min. Only the antisera against S_5 were strong enough for the following experiments.

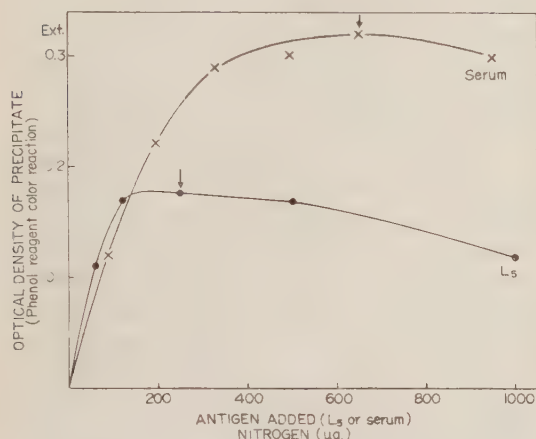


FIG. 4. Quantitative precipitin curves of anti- S_5 sera with L_5 and rat serum. Arrows indicate the maximum precipitates.

By Ouchterlony agar diffusion technique it was shown that anti- S_5 sera formed about four obvious precipitin lines with the corresponding antigen. The antisera reacted not only with S_5 but with L_5 giving three strong lines and with rat serum giving one or two obvious lines. It reacted also with S_6 and L_6 though the reaction was faint. See Fig. 3-A.

By immunoelectrophoresis as shown in Fig. 3-B (with a photograph) the anti- S_5 sera gave at least sixteen lines with S_5 , about eleven lines with L_5 and about eight lines with rat serum. Among the lines with S_5 in the figure can be recognized an especially strong and short line with a steep curvature precisely located at the position corresponding to the α -globulin of serum. This line is never seen either among the lines with serum or with L_5 .

In order to remove the antibodies against L_5 and the rat serum the antisera were absorbed with those antigens. For an appro-

priate absorption the optimal amount of the antigens to be added was determined from a quantitative precipitin reaction curve established by Heidelberger (8). The curves are shown in Fig. 4, from which it was learned that the addition of $240\ \mu\text{g.}$ and $640\ \mu\text{g.}$ of L_5 or serum respectively to each 2.0 ml. portion of the antisera formed a maximum precipitate. With those amounts of the antigens the antisera were absorbed.

As shown in Fig. 3-C and D this treated antiserum reacted only with S_5 but not at all with L_5 or serum. It formed one or may be two very specific precipitin lines with S_5 antigen. As compared with Fig. 3-B all lines disappeared except that specific line which was previously mentioned as a strong, short line with a steep curvature located at α -globulin position.

Electrophoretic Isolation of the Specific Antigen

—The starch block electrophoresis technique was successfully used for the isolation; 2.5 ml. portion of concentrated S_5 (about 7% protein) was applied to a starch block in 0.05 M veronal buffer of pH 8.6. The size of the starch block was 5 cm. \times 2 cm. \times 30 cm. and a

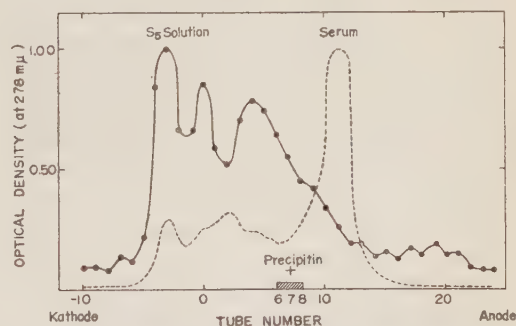


FIG. 5. Electrophoresis of S_5 and serum in starch block (Highest O. D. is accounted as 1.00)

current of 2.5 mA per cm. was given under a voltage gradient $7.0\ \text{volt cm}^{-1}$ for fifteen hours.

After the finish of electrophoresis the block was cut into 1 cm. pieces and each was extracted with 5.0 ml. portion of saline. The precipitin reaction was determined with the

adsorbed antiserum by the usual technique using small test tubes. The amount of extracted protein in each test tube was determined by the O.D. measurement at $278\text{ m}\mu$. One of electrophoresis patterns of S_5 and a rat serum is shown in Fig. 5, where the solid line shows a pattern of S_5 and dotted line is a pattern of rat serum. The precipitin reaction was demonstrated only in test tubes No. 6 to 8, the location of which was exactly same as that of α_2 -globulin of the serum. This strict location of the specific antigen indicates a good agreement with the specific precipitin line which was demonstrated in an immunoelectrophoresis pattern in Fig. 3-B.

DISCUSSION

As seen in Table III it may be pointed out that the content of nucleic acid per mg. of protein nitrogen of tumor cells is higher than that of liver cells. This difference is more clear in DNA content. As described previously this may be explained by that the ascites cells have larger nuclei than do the liver cells. Laird and Miller (9) pointed out that the DNA content per mg. of protein nitrogen in the nucleus of the cells of hepatoma induced by 2-acetylaminofluorene is about twice higher than that of liver cell nucleus while the RNA content is about the same. They also indicated that the RNA content of the mitochondrial fraction is about four times higher in the hepatoma cells than in liver cells. On the other hand Schneider *et al.* (10) did not observe the significant change of the nucleic acid content of rat liver cells during feeding rats with 2-methyl- or 3'-methyl-4-dimethylaminobenzene.

The nucleic acid contents in tumor cells may vary tumor to tumor or depending on the stage of the induction of the tumor by the azo dyes.

The nucleoprotein fractions (see Table III) obtained from the livers or ascites cells revealed no significant difference in the contents of nucleic acids although the number of cases investigated is not large enough. However the optical density of L_6 is much

higher than that of S_6 (Table II). This may suggest the difference in nucleic acid composition between both of the fractions.

Either S_6 or L_6 showed a high content of DNA. This may indicate that the fractions came, if not all, mainly from nucleus of the cells.

S_5 or L_5 fractions never gave a typical green color of pentose but developed a purple color when the Bial's orcinol reaction was applied. It seemed to be a typical color of sialic acid although we did not measure the acid quantitatively. This means that those fractions contain glycoproteins in some extent.

The ultracentrifugal patterns of the fractions were surprisingly simple indicating a main peak which migrates as fast as the serum albumin does, although each fraction showed a very small amount of more rapidly sedimentating fractions. Peterman *et al.* (11, 12) studied the nucleoprotein fractions isolated from rat livers and hepatomas and demonstrated about five sharp peaks of high sedimentation constants. The discrepancy between their results and ours is hard to explain but one of the reasons could be attributed to proteolytic or nucleic acid depolymerizing action in our sample which had been kept in the refrigerator at 3°C for several days. However the sedimentation constants of most parts of our fraction are around 4. If the failure to find some components of high sedimentation rates is attributed to depolymerization we have to assume the sedimentation rates of the depolymerized products are always around 4. It might not be reasonable to assume such uniform split products.

The presence or absence of specific antigens in tumor cells has been demonstrated by many ways so far, *e.g.*, complement fixation (13, 14), radioisotope-labelled antibody techniques (15, 16), fluorescent antibody technique (17) and so on. However the precipitin technique is one of the most useful methods which is more precise and quantitative if an antiserum of high enough titre can be obtained. Korngold (4) and Korosteleva (5) have shown the presence of specific antigen

by the agar diffusion method as previously mentioned. As shown in Fig. 3-A and B anti-S₅ sera reacted strongly with rat liver protein (L₅) and rat serum. This fact suggests that the ascites cells contain several common antigens to the liver and serum proteins. Since the ascites cells were very carefully washed about ten times with a large volume of saline only insignificant amount of serum proteins may retain unless the serum proteins bind firmly at the surface of the ascites cells. It may be most reasonable to assume that the plasma proteins are present inside of the ascites cells.

In Fig. 3-A we can observe that some of the precipitin lines of L₅ are confluent with the lines of serum, and some other lines are confluent with the lines of S₅ fraction. This fact may indicate that the liver cells also contain some common antigen to the rat serum. Nevertheless, L₅ fraction might possibly be contaminated with plasma proteins in some extent even though the livers were extensively perfused. Therefore it would not surely be concluded by this experiment that the plasma proteins may exist in the liver cells.

In Fig. 3-A few lines are observed at the S₅ cup and one of them which is closest to the S₅ cup is never seen at other cups. This line seems to correspond to a unique line in the immunoelectrophoretic patterns (Fig. 3-B) as previously mentioned. This line also corresponds to a characteristic line(s) demonstrated with the absorbed antisera as seen in Fig. 3-C, D.

The immunoelectrophoresis and the agar diffusion techniques are very fine and sensitive methods. However there still remain some uncertainty. Because of this uncertainty it may not be concluded definitely that there exists a specific protein in the ascites hepatoma cells but the very clear cut pattern shown in Fig. 3-C, D may strongly suggest its existence.

The nucleoprotein fractions S₆, L₆ induced only very weak antisera in the rabbit so that the immunochemical analysis of those fractions has not yet been made. Those fractions showed a weak but obvious cross reaction

with anti-S₅ sera as seen in Fig. 3-A, but S₆ (L₆) is unavoidably mixed with S₅ (L₅) *vice versa*, therefore the interpretation of the cross reaction would not be very meaningful. With the absorbed anti-S₅ sera the nucleoprotein fractions (S₆, L₆) did not show any precipitin lines.

The isolation of a specific protein by starch block electrophoresis has been done successfully but the available quantity of the protein isolated was very small.

An attempt has been made to purify this protein by ammonium sulfate and rivanol precipitation. It was known that the protein was precipitated by half saturation of ammonium sulfate but not by one third saturation. The rivanol precipitation method was not successful. Further purification of this protein is under investigation.

SUMMARY

Proteins were extracted from rat transplanted hepatoma cells (AH 49 Sasaki Institute, Tokyo) and from rat livers and some chemical and immunochemical comparison was made between the proteins from both sources. Extraction was performed with 0.1 M acetate buffer of pH 4.8 followed by successive extraction with 0.1 M phosphate buffer of pH 7.0. The results are summarized as below:

1. The amount of protein nitrogen extracted at pH 4.8 from the ascites cells (S₅ fraction) was 12.5 per cent of total nitrogen of the tissue and that from liver (L₅ fraction) was 17.7 per cent.

2. The amount of protein nitrogen extracted successively at pH 7.0 was 19.4 per cent of the total nitrogen of the ascites cells (S₆-fraction) and 13.9 per cent of the liver cells (L₆ fraction).

3. The nature of the absorption spectra of S₅(L₅) was essentially the same as that of serum albumin while that of S₆(L₆) was the same as that of nucleic acids purified from yeast.

4. Optical density at 260 and 278 mμ of the fractions of 1 mg. N/ml. concentration was measured. The density of L₆ was significantly higher than that of S₆.

5. Electrophoretic analysis of each fraction revealed 8 to 10 peaks. The patterns obtained were fairly reproducible.

6. Ultracentrifugal analysis indicates that each fraction consists of a main component of about 4 S and lesser amounts of components of 6-10 S.

7. The rabbits were immunized with the fractions by the aid of Freund's adjuvant. Although the antigenicity of the fractions was weak, S₅ gave antisera of fairly high titer.

8. Anti-S₅ precipitin sera thus obtained reacted markedly not only with S₅ but also with L₅ and rat serum in Ouchterlony's agar gel diffusion. S₆ and L₆ fractions also revealed cross reactions with the anti-S₅ sera although the reaction was faint.

9. Immunoelectrophoretic analysis using anti-S₅ sera indicated at least sixteen precipitin lines with S₅, about eleven lines with L₅ and seven lines with rat serum.

10. Immunoelectrophoretic and agar diffusion patterns of S₅ demonstrated a characteristic precipitin line(s) which was found in the patterns neither of L₅ nor of rat serum.

11. The antisera against S₅ were absorbed with the appropriate amount of L₅ and rat serum. The treated sera reacted only with S₅ giving a clear precipitin line but not at all with L₅ and rat serum.

12. This specific protein was isolated successfully by means of starch block electrophoresis. The protein located strictly at the position of α_2 -globulin.

The authors express their deep appreciation to Prof. N. Shimazono, Department of Biochemistry,

Faculty of Medicine, University of Tokyo, for his kind advice, also to Dr. Robert Pennell, School of Public Health, University of Harvard, for his valuable suggestion, to this study.

REFERENECS

- (1) Freund, J., and Bonanto, M. V., *J. Immunol.*, **48**, 325, (1944)
- (2) Ouchterlony, O., *Acta Pathol. Microbiol. Scand.*, **32**, 231 (1953)
- (3) Grabar, P., and Williams, C. A., Jr., *Biochim. et Biophys. Acta*, **17**, 67 (1955)
- (4) Korngold, L., *Cancer Res.*, **19**, 956 (1956)
- (5) Korosteleva, T. A., "Pathogenesis and Immunology of Tumors" Edited by G. V. Vygodchikov, Pergamon Press Ltd, p. 142-151 (1959)
- (6) Mejbbaum, W., *Z. Physiol. Chem.*, **258**, 117 (1939)
- (7) Dische, Z., and Shwarz, K., *Mikrochim. Acta*, **2**, 13 (1937)
- (8) Heidelberger, M., and Kendall, F. E., *J. Exptl. Med.*, **95**, 333 (1952)
- (9) Laird, A. K., and Miller, E. C., *Cancer Res.*, **13**, 464 (1953)
- (10) Schneider, W. C., Hogeboom, G. H., Schelton, E., and Striebach, M. J., *Cancer Res.*, **13**, 285 (1953)
- (11) Peterman, M. L., Mizer, N. A., and Hamilton, M. G., *Cancer Res.*, **16**, 620 (1956)
- (12) Peterman, M. L., *Cancer Res.*, **13**, 372 (1953)
- (13) Rapport, M. M., and Graf, L., *Cancer*, **8**, 538 (1955)
- (14) Rapport, M. M., and Graf, L., *Proc. Am. Assoc. Cancer Res.*, **2**, 141 (1956)
- (15) Lawrence, J. H., and Tobias, C. A., *Cancer Res.*, **16**, 185 (1956)
- (16) Bale, W. F., Spar, I. L., Goodland, R. L., and Wolfe, D. W., *Proc. Soc. Exptl. Biol. Med.*, **89**, 564 (1955)
- (17) Weiler, E., *Z. Naturforsch.*, **116**, 31 (1956)

Biochemical Studies on Gingival Pigmentation in Children

By KAICHIRO KURODA, IWAO KARAZUMI, MASAOKI FUJINO, ZINSAKU TANI,
AKIRA NAGAI and TAKEO MATSUZAWA

(From the Department of Biochemistry, School of Medicine, Tokushima University, and the Research
Institute for Enzymology, Tokushima University, Tokushima)

(Received for publication, March 18, 1961)

The nutritional survey of school children in Japanese mountain villages showed that a band-shaped pigmentation at the gingiva was observed in about 50 per cent of the children examined. This peculiar pigmentation has no signs of inflammation and shows diffuse light brown to dark brown color with no elevation of gingival mucosa and this condition was termed "juvenile gingival pigmentation," which can be differentiated from gingivitis.

The present paper reports the results of biochemical studies on the etiology of the gingival pigmentation and shows that the condition results from the deficiency of vitamin C. At the same time, the effect of vitamin C on tyrosinase, which plays an important role in melanin formation, was examined. Re-examination of vitamin C requirement of Japanese population is also involved.

EXPERIMENTALS AND RESULTS

Occurrence of the Gingival Pigmentation—The gingival pigmentation is band-shaped and is of light brown to dark brown color, as illustrated in Photo 1. The gingival pigmentation is mostly observed at both gums or mandibular gingiva and the cases in which the pigmentation was found only at maxillary gingiva are quite few (Table I). The percentage of the incidence of the gingival pigmentation is highest in infants of about 6 years of age and the incidence decreases gradually in higher age group (Fig. 1). As for the social distribution of the juvenile gingival pigmentation, the incidence is highest in large cities, and follows in the order of fishing, mountainous, and farm villages (Table II).

TABLE I

Location of Juvenile Gingival Pigmentation

Location	Male		Female	
	No. of patients	%	No. of patients	%
Maxillary gingiva	70	4.5	61	3.8
Mandibular gingiva	593	37.8	677	41.7
Both gingivae	904	57.7	887	54.5
Sum	1,567	100.0	1,625	100.0
Total number of persons examined	3,076		3,167	

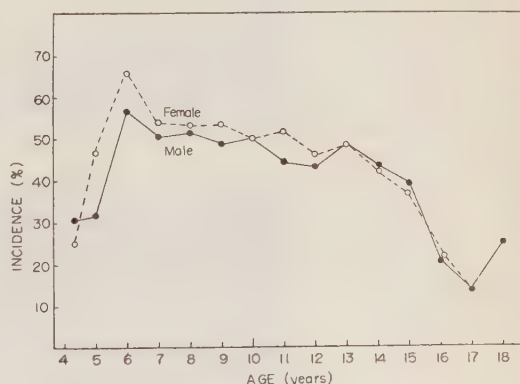


FIG. 1. Incidence of juvenile gingival pigmentation in relation to age.

Relationship between the Gingival Pigmentation and Vitamin C—According to Ralli and Sherry's method (1), 100 mg. of vitamin C was injected intravenously, urine excreted during the following 3 hours was collected, and vitamin C in the urine was determined by the method of Nagayama (2). This vitamin C saturation test was performed on children with the gingival pigmentation, with

TABLE II
Social Distribution of Juvenile Gingival Pigmentation

Social circumstances	Male			Female		
	Total number of persons examined	Number of patients	%	Total number of persons examined	Number of patients	%
City	628	346	55.1	632	373	59.0
Fishing village	525	266	50.7	552	312	56.5
Mountain village	1,261	519	41.2	1,218	564	46.3
Farm village	2,531	1,032	40.8	2,586	1,033	39.9

gingivitis and with both of them in comparison with normal children. The result is shown in Table III, which shows that the saturation degree of vitamin C in children with gingivitis or gingival pigmentation is much lower than that of normal children. This indicates that the gingival pigmentation likely occurs in vitamin C-deficient children.

When 12 cases of children with gingivitis were fed on the routine meal in their families, the gingivitis never disappeared and furthermore brown pigmentation appeared on gums of 2 children 6 months later. However, when 50mg. of vitamin C was administered orally every day besides common meal of the family to children with gingivitis, gingivitis disappeared and the gingival pigmentation did not occur. This shows that vitamin C can prevent the gingival pigmentation.

The coloration degree of the gingival pigmentation can be expressed macroscopically

by —, ±, +, ++ and ###. For the sake of convenience of mathematical treatment, the number 0, 0.5, 1, 2 and 3 was given to each sign, respectively, and the coloration degree of the gingival pigmentation of individual person was expressed by the sum of the coloration numbers of maxillary and mandibular gums. When 100mg. of vitamin C was administered orally every other day to children with the gingival pigmentation, coloration of the gingiva due to pigment decreased gradually and reached 61.1 in 6 months, compared with the initial coloration of 100 (%) prior to the administration of vitamin C. The control group of children with the gingival pigmentation, to whom no vitamin C was administered, showed only a slight decrease in coloration of the gingiva, and 6 months later the coloration was still 90.8, as against the initial coloration of 100 (Fig. 2). When, however, the administration of vitamin C in

TABLE III
Relationship between Gingival Findings and Saturation Degree of Vitamin C

Gingival findings		Amount of Vitamin C		F	F	Significance of the difference from healthy persons
Pigmentation	Gingivitis	Mean (mg.)	Standard deviation (mg.)			
—	—	17.1	13.9	—	—	—
—	+	9.1	12.7	4.30	4.02 ($\alpha=0.05$)	Significant
+	—	8.6	10.5	5.16	4.08 ($\alpha=0.05$)	Significant
+	+	7.8	8.2	7.88	7.31 ($\alpha=0.01$)	Significant

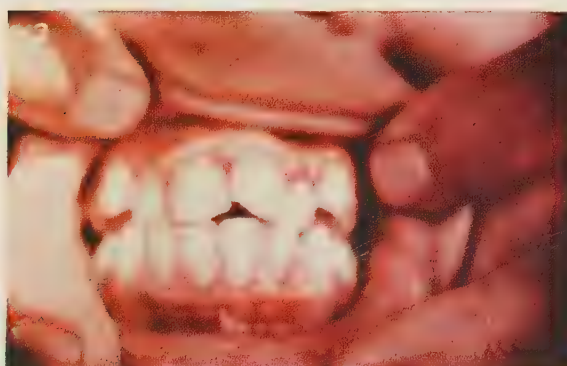
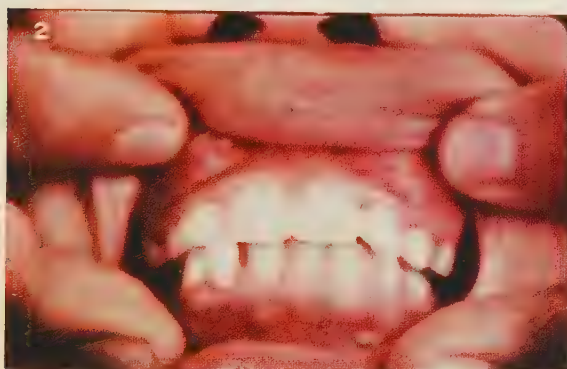


PHOTO. 1. Pictures of juvenile gingival pigmentation.
 1. Gums of healthy person
 2 and 3. Gums of juvenile gingival pigmentation.

whether the therapeutic effect of vitamin C is attributable to the nonspecific decoloration due to the reducing effect of vitamin C or to an unknown specific action of vitamin C. In order to elucidate this problem, effect of vitamin C upon tyrosinase, which plays an important role in melanin formation, was examined.

a) *Preparation of Tyrosinase*—Tyrosinase was partially purified from crusts of potatoes by Kubowitz method (3). This purification procedure is illustrated in Scheme I. One mg. of the purified enzyme showed 60 units by the author's method.

b) *Substrate Solution*—Forty mg. of DL-2, 4-dihydroxy-phenylalanine (DOPA) was dissolved in 20 ml. of distilled water, heated in a boiling water bath, and 2 mg./ml. DOPA solution was obtained.

c) *Solution of Reduced Vitamin C*—A definite mg. of L-ascorbic acid was dissolved in 100 ml. of redistilled water and immediately used for the experiment.

d) *Measurement of Activity*—Twelve ml. of the enzyme solution was placed in a cuvette and 0.3 ml. of reduced vitamin C solution and 0.5 ml. of DOPA solution were added to it. The enzymatic reaction was carried out within the cuvette of Coleman spectrophotometer. Using a stop-watch, the time required for the optical density to begin to increase and the value of the optical density at unit time were measured at 485 $m\mu$, where the maximum light absorption of DOPA chrome was observed. Since the spectrum of ultraviolet absorption of reduced vitamin C was different from those of DOPA and tyrosinase, the amount of reduced vitamin C was measured at 260 $m\mu$ with time.

DOPA-DOPA chrome reaction by tyrosinase was inhibited for a certain period of time by the addition of reduced vitamin C. As shown in Fig. 3, the length of the lag period was in correspondence to the concentration of reduced vitamin C, namely, a lag period of 2.5, 3.5 and 4.5 minutes with a concentration of reduced vitamin C of 1.0, 1.4 and 1.8 mg./dl., respectively, but the decrease of reduced vitamin C proceeded at the same

rate. The enzymatic reaction seems to take place immediately after reduced vitamin C has disappeared, and in each case the enzymatic reaction proceeded at about the same rate as that of the control.

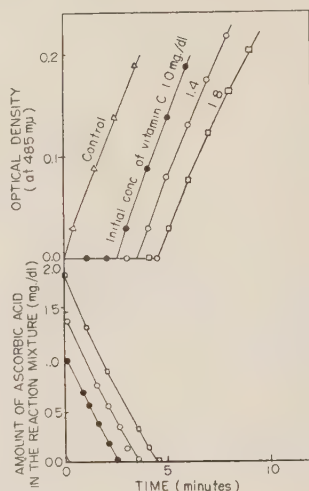


FIG. 3. Relationship between the inhibition of enzymatic activity by vitamin C and the initial concentrations of reduced vitamin C.

Concentrations of DOPA and tyrosinase, 2 mg./ml. and 10 mg./dl., respectively.

When the concentration of the substrate and reduced vitamin C was kept constant and the concentration of tyrosinase was varied to 5, 2.5, and 1.25 mg./dl., the inhibition period became 2, 4, and 8.5 minutes, as shown in Fig. 4. The amount of reduced vitamin C decreased rapidly at a high concentration and slowly at a low concentration of the enzyme. At any rate, the enzymatic reaction was restored when reduced vitamin C disappeared from the reaction mixture.

When the concentrations of reduced vitamin C and tyrosinase were kept constant and the concentration of DOPA was varied to 1.0, 0.5 and 0.25 mg./dl., the amount of reduced vitamin C in the reaction mixture decreased rapidly at a high concentration and slowly at a low concentration of the substrate. Just like the above cases the inhibition came to end and the enzymatic reaction was restored just when reduced vitamin C had disappeared (Fig. 5).

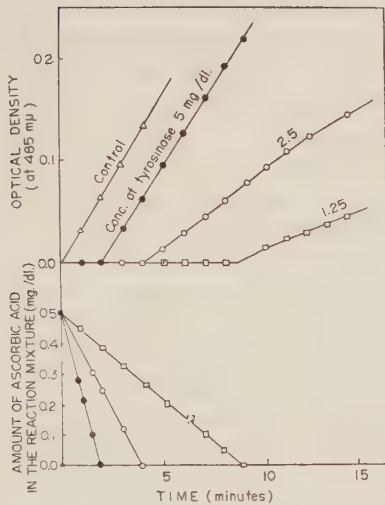


FIG. 4. Relationship between the inhibition of enzymatic activity by vitamin C and the concentration of tyrosinase.

Concentrations of DOPA and ascorbic acid, 2 mg./ml. and 0.5 mg./dl., respectively.

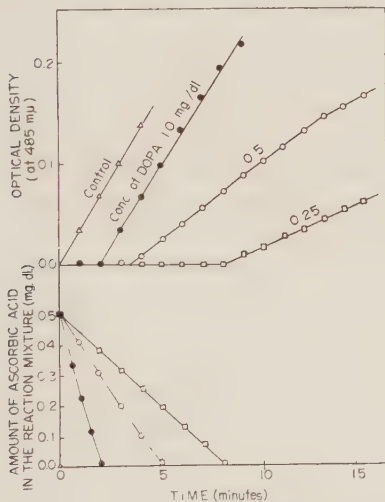


Fig. 5. Relationship between the inhibition of enzymatic activity by vitamin C and the concentration of substrate (DOPA).

Concentrations of ascorbic acid and tyrosinase 0.5 mg./dl. and 5 mg./dl., respectively.

It was confirmed that glutathione and cysteine, both of which are important reducing substances in a living system as is ascorbic acid, also have an inhibitory effect on DOPA-DOPA chrome reaction.

Thus, the mechanism of the formation of gingival pigmentation by vitamin C-deficiency could be well explained by the above experiments. Through the lack of vitamin C, the inhibitory effect of vitamin C on DOPA-DOPA chrome reaction decreases and the reaction proceeds in a living body to form melanin pigment more easily.

Re-examination of Vitamin C Requirement of Japanese Population—Since the gingival pigmentation due to vitamin C-deficiency is frequently observed at the present time in Japanese school children, it is considered to be quite necessary to re-examine the dietary allowance of vitamin C in Japanese population, especially in children. By the consecutive oral administration of various test doses of vitamin C, the state of vitamin C saturation was investigated from vitamin C concentration in blood, and dietary allowance of vitamin C in adult and child was also examined. Vitamin C in whole blood was determined by dinitrophenylhydrazine method (4).

Oral administration of various test doses of vitamin C was made with strictly vitamin C-free diet, in schoolboys aged 10 to 12 years and adults of 23 to 32 years (Fig. 6).

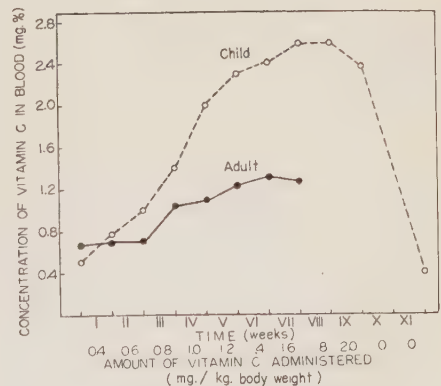


FIG. 6. Relationship between vitamin C administration and concentration of vitamin C in blood.

In the 1st week, vitamin C of 0.4 mg. per kg. was given daily and the vitamin C in blood was determined on the last day of the week. In the 2nd week, vitamin C of 0.6 mg./kg. was given daily and vitamin C in blood was determined on the last day of the week, and so on.

In adults, the requirement of vitamin C to keep the blood level of vitamin C in a state of saturation was 1.2 to 1.6 mg. (mean value, 1.4 mg.) per kg. body weight, and the saturated concentration of vitamin C in blood was 1.2 to 1.3 mg./dl.. In children, the requirement of vitamin C to keep the blood level of vitamin C in a state of saturation was 1.6 to 1.8 mg. per kg. of body weight and the saturated concentration of vitamin C in blood was 2.5 to 2.6 mg./dl..

Thus, in children, the amount of vitamin C necessary to keep the saturated level of blood vitamin C is higher than that of adults, and the saturated level of blood vitamin C is also higher in children than in adults. This fact implies that children become more easily deficient in vitamin C than adults, thus explaining the reason for the markedly high incidence of gingival pigmentation in children than in adults, which is considered to be caused by the lack of vitamin C.

For the dietary allowance, it might be more reasonable to take the amount of vitamin C necessary to keep the saturated level of blood vitamin C rather than the amount of vitamin C necessary to prevent scurvy. Therefore, 1.4 mg. of vitamin C per kg. of body weight might be recommended as the daily requirement of a Japanese adult and 1.7 mg. of vitamin C per kg. of body weight as that of a Japanese child.

DISCUSSION

Besides the juvenile gingival pigmentation described herein, various kinds of gingival pigmentation have been reported so far. One of the most frequent cases of pigmentation is the condition due to the intoxication by heavy metals such as mercury (5). In the case of the intoxication by heavy metals, gingival pigmentation is observed at the peripheral part of the gingiva close to dental surface and it is the pigmentation of metal granules. In the case of Addison's disease, pigmentation of grey to black-brown color is often observed at oral mucosa, especially at buccal mucosa. The mucosa pigmentation is maculate and skin pigmentation is also ob-

served at the same time (6). As a physiological pigmentation, Reiche (7) reported pigmentation of mouth mucosa of Negro, Indian, Arabian, and Chinese. In Japanese, Okuguchi (8) and Mine (9) reported physiological pigmentation of light brown to dark brown color in the mouth and in a few cases gingiva was also reported to be included. Fujibayashi (10) examined the gums of many Japanese and reported that gingival pigmentation was observed in about 5 per cent of the people examined, and Miyoshi (11) confirmed the pigment of gingival pigmentation to be melanin.

However, these physiological gingival pigmentations are observed in adults or in aged persons, while the juvenile gingival pigmentation herein described is observed in the younger generation of pre-school to school age, and the local findings and incidence are different from them.

By nutritional studies, etiology of the juvenile gingival pigmentation was attributed to the deficiency of vitamin C. Reduced vitamin C has an effect of inhibiting the DOPA-DOPA chrome reaction, probably attributable to the oxidation of reduced vitamin C. In the absence of vitamin C, DOPA-DOPA chrome reaction may proceed more easily in a living body to form melanin pigment. In order to explain this inhibition phenomenon of vitamin C, a possible mechanism for the reaction was assumed.

DOPA reacts with $1/2 O_2$ catalyzed by tyrosinase and DOPA-quinone is formed, which in turn forms a leuco compound immediately by the intramolecular rearrangement. The leuco compound is immediately converted to DOPA-chrome by a spontaneous oxidation. When, however, reduced vitamin C (ascorbic acid) is present in the reaction mixture, DOPA-quinone may again be converted to DOPA by ascorbic acid, as long as ascorbic acid is present in the reaction mixture, and the reaction of DOPA-quinone to leuco compound ceases. At the same time, ascorbic acid is converted to dehydroascorbic acid. Therefore, when ascorbic acid is completely oxidized, the original DOPA-DOPA

chrome reaction may be restored.

The inhibitory effect of reduced vitamin C on tyrosinase activity was already reported by Lerner (12) and the fact that the enzymatic oxidation of DOPA does not proceed in the presence of reduced vitamin C was also discussed, but the mechanism of this inhibition by reduced vitamin C was not well elucidated. Mizuno (13) in his interesting study assumed that the inhibition of tyrosinase by reduced vitamin C was attributable to the reduction of copper in the tyrosinase by reduced vitamin C. However, when a large amount of reduced vitamin C is added to the tyrosinase reaction, tyrosinase is used for a long period of time for the oxidation of reduced vitamin C and tyrosinase catalyzes the original DOPA-DOPA chrome reaction with the same reaction velocity as that of the control reaction only after reduced vitamin C is consumed from the reaction mixture (Fig. 3). From this fact, it is considered that the reduction of copper in tyrosinase might not be the mechanism of the inhibitory effect of vitamin C.

Both tyrosinase and ascorbic acid are present in a normal body. If there is a state of vitamin C deficiency, the inhibitory effect of ascorbic acid on tyrosinase described above may decrease and the formation of melanin may then be accelerated. This is considered to be the cause for the abnormal gingival pigmentation.

SUMMARY

1. Peculiar band-shaped pigmentation of light brown to dark brown color was observed at the gingiva in high percentage among Japanese children and the term of "juvenile gingival pigmentation" is proposed.

2. From the incidence of gingival pigmentation in vitamin C-deficient children, as shown by vitamin C saturation test, as well as by preventive and therapeutic effect of vitamin C on gingival pigmentation, etiology of the juvenile gingival pigmentation is attributed to the deficiency of vitamin C.

3. In connection with this problem, the effect of vitamin C on tyrosinase, which plays

an important role in melanin formation, was discussed. When ascorbic acid is present in the reaction mixture, DOPA-quinone formed by tyrosinase from DOPA is considered to be reconverted to DOPA by ascorbic acid and the reaction of DOPA to DOPA-chrome ceases.

4. As the daily requirement of vitamin C in Japanese adult and child, 1.4 mg. and 1.7 mg. per kg. of body weight, respectively, may be recommended from the experiment to keep the saturation level of blood vitamin C. In children, the amount of vitamin C necessary to keep the saturated level of blood vitamin C is higher than that in adults, and the saturated level of blood vitamin C is also higher in children than that in adults. This fact implies that children become more easily deficient in vitamin C than adults and also explains the reason for the markedly high incidence of the gingival pigmentation due to vitamin C-deficiency in children than in adults.

The authors wish to express their heartfelt gratitude to Dr. K. Kodama, President of Tokushima University, for his continued encouragement and interest during the course of this work.

REFERENCES

- (1) Ralli, E. P., and Sherry, S., *Medicine*, **20**, 251 (1941)
- (2) Nagayama, T., Tomoi, T., and Soraku, S., *J. Japan. Biochem. Soc.*, **14**, 58 (1939)
- (3) Kubowitz, F., *Biochem. Z.*, **292**, 221 (1937)
- (4) Takahashi, T., *Vitamin*, **8**, 203 (1955) in Japanese
- (5) Baum, H. B., *Det. Cosmos.*, **74**, 247 (1934)
- (6) Nobel, *Wien. Med. Wschr.*, **76**, 729 (1926)
- (7) Reiche, F., *Hamburg. Med. Klin.*, **7**, 206 (1924)
- (8) Okuguchi, M., *Hifuka oyobi Hinyokika Zasshi* (in Japanese), **27**, 646 (1927)
- (9) Mine, M., *Hifuka oyobi Hinyokika Zasshi* (in Japanese), **28**, 325 (1928)
- (10) Fujibayashi, T., *Kokuhyo Gakkai Zasshi* (in Japanese), **12**, 297 (1938)
- (11) Miyoshi, Y., *Rinsyo Seika* (in Japanese), **9**, 893 (1937)
- (12) Lerner, A. B., *Advances in Enzymology*, **14**, 92 (1953)
- (13) Mizuno, K., *Acta Soc. Ophthal. Japon.*, **58**, 1130 (1954)

The Reciprocal Relationship between Melanization and Tyrosinase Activity in Melanosomes (Melanin Granules)

By MAKOTO SEIJI and THOMAS B. FITZPATRICK

(From the Research Laboratories of the Department of Dermatology of the Harvard Medical School at the Massachusetts General Hospital, Boston 14, Massachusetts, U. S. A.)

(Received for publication, March 18, 1961)

In mammals, melanin pigment is synthesized in a specific cell, the melanocyte, by the action of a copper-containing oxidase, tyrosinase (1-3). This enzyme catalyzes the aerobic oxidation of tyrosine, the precursor of melanin, to a monomer, 5,6-dihydroxyindole; this monomer, in turn, is transformed into a large polymer which is probably attached through its quinone linkages to the amino or sulfhydryl groups of the protein matrix of the pigment granule. It has been shown that mammalian tyrosinase is attached to a specific cytoplasmic particle (4, 5). The name "melanosome" has been proposed for these distinctive, enzymatically active particles which are the site of melanin formation and are found only within the cytoplasm of the melanocyte (6). Melanosomes have been shown by biochemical and electronmicroscopic studies to be different from mitochondria (6). Electronmicroscopy has revealed the morphology of melanosomes and melanin granules (7-10): Birbeck and Barnicot (11) and Seiji, Fitzpatrick and Birbeck (6) have reported that a series of steps can be seen in the formation of melanin granules within the melanocyte.

Recently we have become aware that there appears to be an inverse relationship between amount of tyrosinase activity and degree of melanization within melanosomes. The experiments described in this paper were performed in an effort to clarify this relationship. Melanosomes were obtained from two types of mammalian melanoma and from the retinal pigment-epithelium of the chick embryo. A decrease in reaction velocity was

found to be associated with *in vitro* melanization of isolated melanosomes.

EXPERIMENTAL METHODS

Preparation of Large-Granule Suspension from Mouse Melanoma—B-16 and Harding-Passey mouse melanoma were serially transplanted in C-57-strain mice and Swiss-strain mice respectively. The entire, actively growing melanoma was excised when the diameter reached 1-1.5 cm. The tumor was promptly homogenized in 0.25 M sucrose solution at about 0°C. All subsequent processing took place at a temperature of about 3°C. The homogenate was centrifuged at $700 \times g$ for 10 minutes. The resulting "low-speed" supernatant, when centrifuged at $11,000 \times g$ for 10 minutes, yielded a sediment which was resuspended in 0.25 M sucrose and recentrifuged at $15,000 \times g$ for 10 minutes. The sediment thus obtained was again suspended in 0.25 M sucrose to make the "large-granule suspension" used as starting material for density-gradient centrifugation in our experiments.

Preparation of Large-Granule Suspension from Retinal Pigment-Epithelium of Chick Embryos.—Retinal pigment epithelium, dissected from the eyes of Rhode-Island-Red chick embryo, was homogenized in 0.25 M sucrose solution. The homogenate was centrifuged at $300 \times g$ for 5 minutes and two more successive centrifugations were carried out at the same speed with the respective supernatants. The last supernatant was centrifuged again at $11,000 \times g$ for 10 minutes. The resulting sediment was then resuspended in 0.25 M sucrose solution and recentrifuged at $15,000 \times g$ for 10 minutes. The sediment thus obtained was suspended in 0.25 M sucrose to form the "large-granule suspension" used as the starting material for density-gradient centrifugation in our experiments.

Preparation of the Specific-Gravity-Gradient Tubes.—Tubes of the Spinco swinging-bucket rotor (SW 39-L) were prepared by layering 0.5 ml. of eight different concentrations of sucrose solution in serial order, with

the most concentrated layer at the bottom of the tube (12). They were then allowed to stand overnight (about 18 hours) so that the gradient might become smooth. The sucrose concentrations used for separation of melanosomes (Fig. 1 A) were 2.6 *M*, 2.4 *M*, 2.2 *M*, 2.0 *M*, 1.8 *M*, 1.6 *M*, 1.55 *M* and 1.5 *M*. Immediately before centrifugation, 1 ml. of freshly prepared large-granule suspension from mouse melanoma or pigment-epithelium of the chick embryo was layered carefully over the top of each tube.

Isolation of the Melanosome Fraction after Centrifugation—At the end of centrifugation, the position of the strata in each tube was recorded (Fig. 1 B) and the fraction contained in the bottom of the tube (*i. e.*, between the bottom and a point 1 cm above the bottom) was isolated by means of a specially designed centrifuged-tube cutter (13). The fraction thus obtained constituted the "melanosome suspension."

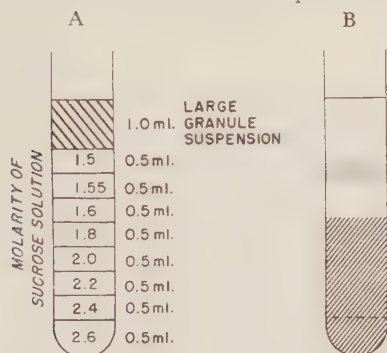


FIG. 1. A. Density-gradient tube before centrifugation, showing the layering of eight different concentrations of sucrose solution and the large-granule suspension (in 0.25 *M* sucrose). The gradient was prepared 18 hours in advance; the large-granule suspension was added immediately before centrifugation.

B. The two opaque regions appeared in the gradient tube after ultracentrifugation at 103,000 $\times g$ for 1 hour. The place where the tube was cut 1 cm. from the bottom is shown by a dotted line.

Determination of Tyrosinase Activity—Tyrosinase was determined manometrically by measurement of the oxygen consumption. L-Tyrosine, L-dopa, and a mixture of L-tyrosine and L-dopa in the 0.1 *M* phosphate buffer at pH 6.8 were used as substrates.

Protein-Nitrogen Determination—Protein was precipitated by adding 10% trichloroacetic acid to each sample. The precipitate was spun down, washed once with trichloroacetic acid and dissolved in *N* NaOH. The nitrogen-content of this alkaline solution was

determined by the micro-Kjeldahl method.

Electronmicroscopy—For electronmicroscopy, suitable aliquots of each preparation were mixed at 0°C with 1% osmic acid tetroxide buffered with acetate-veronal to pH 7.4, centrifuged at 11,000 $\times g$ for 10 minutes, and kept at about 3°C for fixation. After fixation for 2 hours, the sediment was dehydrate in a graded series of ethyl alcohols and allowed to polymerize with Araldite resin. Sections cut with a modified Cambridge rocker-microtome were studied by means of a Siemens Electronmicroscope 1.

Estimation of the Optical Density of the Melanosome Suspension—The optical density of the melanosome suspension was measured by means of a Beckman spectrophotometer, Model DU, in the range between 400 $m\mu$ and 700 $m\mu$.

RESULTS

Comparison of the Color Intensity of Melanosomes Isolated from Mouse Melanoma and from the Retinal Pigment-Epithelium of Chick Embryos—Fig. 2 shows that melanosome suspensions prepared from B-16 mouse melanoma are significantly darker than those prepared from Harding-Passey tumors. At 550 $m\mu$, the optic-

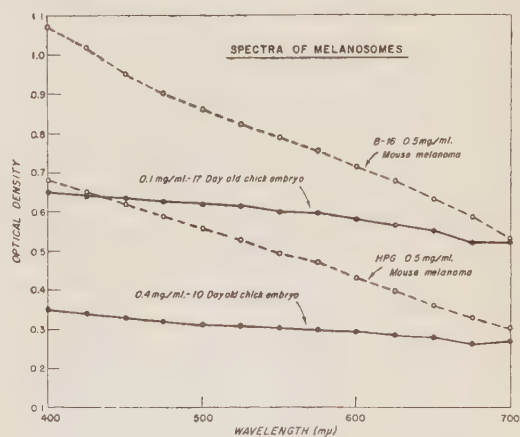


FIG. 2. Absorption spectra of melanosomes from mouse melanoma and retinal pigment-epithelium of Rhode-Island-Red chick embryos. The melanosomes from mouse melanoma and retinal-pigment-epithelium were isolated by the method described in the text, washed several times in distilled water, and resuspended in distilled water. The concentrations of these suspensions are expressed in milligrams of dry weight per milliliter. These readings were taken in a Beckman Model DU spectrophotometer.

al density of the melanosome suspension (0.1 mg. dry weight/ml.) isolated from retinal pigment-epithelium of the 17-day Rhode-Island-Red chick embryo is twice that of suspensions (0.4 mg. dry weight/ml.) isolated from the 10-day chick embryo.

Comparison of the Tyrosinase Activity of Melanosomes Isolated from Mouse Melanoma and from the Retinal Pigment-Epithelium of Chick Embryos—Melanosomes isolated from Harding-Passey mouse melanoma contain three or four times more tyrosinase per milligram of protein-nitrogen than those from B-16 mouse melanoma (Table I). Comparison of melanosome suspensions obtained from 10-day and 17-day chick embryos (Table I) shows that the tyrosinase level of the suspension prepared from the pigment-epithelium of the 10-day chick embryo is twelve times higher than that of the suspension prepared from the corresponding tissue of the 17-day chick.

Some time ago, Miyamoto and Fitzpatrick (14) reported that the level of tyrosinase activity of pigment granules isolated from the retinal pigment-epithelium of

Rhode-Island-Red chick embryos changes as embryonic development advances and that it reaches its highest level in the 10-day chick. The experimental data reported here confirm their observations.

Effect of Incubation in L-dopa on the Tyrosinase Activity of Melanosomes—Keeping in mind the experimental results just described, the following *in vitro* experiment was designed to test the relationship between artificial melanization and changes of tyrosinase activity in melanosomes isolated from Harding-Passey mouse melanoma. These tumors are lighter in color than B-16 mouse melanoma and contain a high level of tyrosinase activity. Fig. 3 shows the experimental procedure.

TABLE I		
<i>Comparison of the Tyrosinase Activity</i>		
Experiment Number	Specific Activity	
	<i>μl. per hour per mg. protein-nitrogen</i>	
	Harding-Passey	B-16
1	1114	210
2	1120	256
3	850	365
4		141
Retinal Pigment-Epithelium		
	10-day chick embryo	17-day chick embryo
1	592	49.5

Comparison of the tyrosinase activity of melanosomes isolated from mouse melanoma and from retinal pigment-epithelium of chick embryos. Tyrosinase activity was estimated respirometrically by measuring the oxygen consumption, using a 10:1 mixture of L-tyrosine and L-dopa as substrate (1.77 μ moles) in the 0.1 M phosphate buffer (pH 6.8).

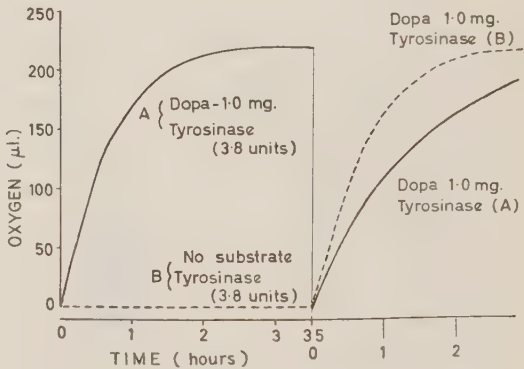


FIG. 3. Effect of dopa incubation on melanosomes isolated from Harding-Passey mouse melanoma. Melanosomes of experimental series A were incubated with L-dopa in Warburg apparatus at 38°C with 0.1 M phosphate buffer at pH 6.8; control group B was incubated without dopa under the same conditions. After the oxygen consumption of the experimental group reached its maximum, 1 mg. of L-dopa was added to the reaction vessels of both groups for measurement of tyrosinase activity.

Suspension A was incubated with 1 mg. of L-dopa. When the oxygen consumption had reached its maximum after 3.5 hours of incubation, the tyrosinase activity of the suspension was measured, using dopa as a substrate. Simultaneously with Suspension A, Suspension B was incubated under identical conditions, but without dopa, so that the tyrosinase activity of the two suspensions

TABLE II

Effect of Incubation in L-dopa on the Tyrosinase Activity of Melanosomes

Expt. No.	Dopa	Reaction Velocity of	Reaction Velocity of	Reaction Velocity of
		Original Melanosomes	Original Melanosomes	Incubated Melanosomes
	gm.	μ l. per minute	μ l. per minute	μ l. per minute
1	0.5	3.8	3.1	2.7
2	0.5	3.4	3.3	3.1
3	0.5		2.8	2.5
4	1.0	4.3	4.1	3.1
5	1.0	4.6	3.8	2.8
6	1.0	3.8	3.4	2.1
7	1.0	3.8	3.1	2.8
8	1.0	3.4	3.3	2.3
9	1.5		3.1	1.1
9	1.2		3.1	1.6
9	0.9		3.1	1.9
9	0.6		3.1	2.4
9	0.3		3.1	2.8

The melanosomes isolated from Harding-Passey mouse melanoma were incubated with L-dopa. After the oxygen consumption reached a maximum, tyrosinase activity was measured by adding 1 mg. of L-dopa and was compared with the tyrosinase activity of untreated melanosomes; incubation was carried out under the same conditions, but without L-dopa.

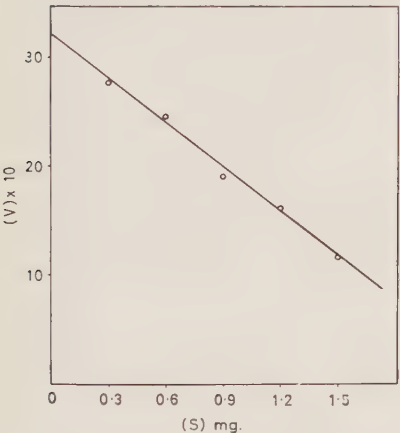


FIG 4. Relationship between dopa concentration (S) and reaction velocity (V) (rate of oxygen consumption, in microliters per minute) in melanosomes incubated in dopa.

could be compared. The color of the reaction mixture of A at the end of incubation was much darker than that of B. As can be seen in Table II, there is a significant decrease in the reaction velocity of tyrosinase

in Suspension A after incubation. A linear relationship between the concentration of dopa in the incubation mixture and the reaction velocity of tyrosinase is shown in Fig. 4. (Experiment No. 9 in Table II).

Electronmicrographs of Melanosomes Which Have Been Melanized in Vitro—In order to demonstrate the morphological changes in melanosomes which follow incubation with dopa, electronmicrographs were taken after incubation of the dopa-treated and control large-granule suspension isolated from Harding-Passey melanoma (Figs. 5 A and B). Fig. 5 B is an electronmicrograph of the intensely electron-dense melanosomes which were never found in control suspensions (Fig. 5 A), but which appeared consistently in fractions which had been incubated with dopa. It is not possible to prepare good electronmicrographs, because of the effect of incubation with or without dopa on the fixation process, but those obtained were sufficiently clear to permit adequate identification of the elements present.

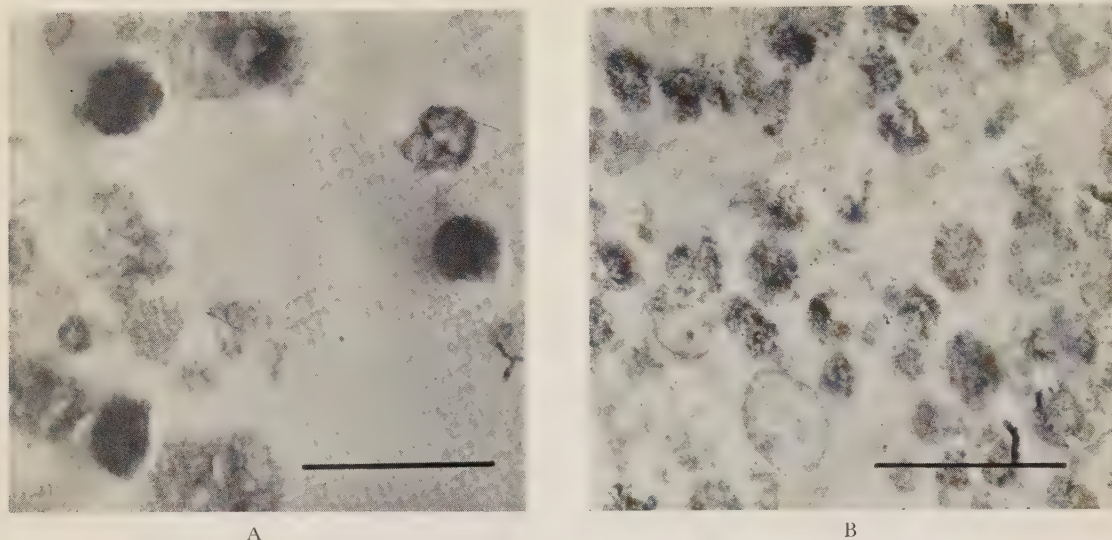


FIG. 5. A. Electronmicrograph of large-granule suspension of Harding-Passey mouse melanoma after incubation without dopa (B in Fig. 3). Melanosomes and mitochondria can be identified. Magnification, $\times 32,000$. The line in the lower part of each illustration denotes the length of 1 micron.

B. Electronmicrograph of large-granule suspension of Harding-Passey mouse melanoma after incubation with dopa (A in Fig. 3). Intensely electron-dense melanosomes were never found in the control suspension, but appear consistently in fractions which have been incubated with dopa. Magnification, $\times 32,000$.

DISCUSSION

The experimental results presented in Fig. 1 and Table I suggest that differences in the specific activity of tyrosinase may be related to degree of melanization of melanosomes. The decrease in reaction velocity following incubation with dopa of melanosomes isolated from Harding-Passey melanoma (Table II) appears to be related to the degree of *in vitro* melanization of melanosomes by incubation in dopa. In melanosomes incubated with dopa for three consecutive periods the tyrosinase activity is reduced 73 per cent below control levels, while in melanosomes incubated for the same period without dopa, the tyrosinase activity is reduced only 7 per cent below initial levels.

The question arises whether the decrease in reaction velocity might be explained by the type of reaction inactivation observed by Nelson *et al.* (15, 16) in preparations of plant tyrosinase. These workers noted that

orthodiphenolase or catecholase activity undergoes early and progressive inactivation as oxidation of the substrate proceeds. In preparations of mammalian tyrosinase, this effect has not been observed with the diphenolic substrate, dopa. The reaction inactivation of plant tyrosinase does not appear to be due to products known to be formed during the oxidation of catechol, but occurs at the time when catechol is oxidized.

The linear relationship between the concentration of dopa in the incubation mixture and the reaction velocity of tyrosinase clearly shows that the relationship between dopa concentration and tyrosinase activity is inverse. The same inverse relationship between enzyme activity and substrate concentration is found in melanosomes which have been incubated with tyrosine instead of dopa. Thus the reduction in the reaction velocity of tyrosinase (as shown in activity units of Hogeboom and Adams (17)) after incubation in tyrosine or dopa would appear

to result from a blocking of the active centers on the enzyme rather than from inactivation of the reaction. It is likely that the centers of tyrosinase activity in the melanosome are blocked by the quinonoid intermediates (dopa-quinone, indole-5,6-quinone) in a chemical process similar to tanning and that during melanization melanosomes are gradually converted from enzymically active particles (early stages) into masses of inert melano-protein (Fig. 6).

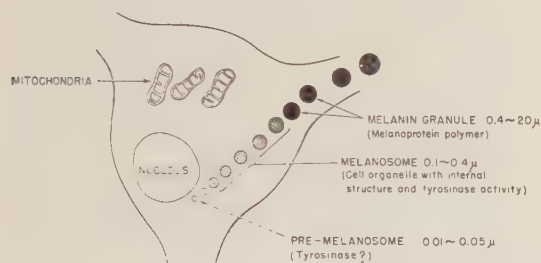


FIG. 6. Diagram showing development of melanin granules in the melanocyte. Melanin granules are distinctive cytoplasmic constituents of the melanin-forming cell and have their own characteristic enzyme (tyrosinase). The term "melanosome" is proposed for the distinctive, enzymatically active particle which is the site of melanin formation and is located within the cytoplasm of the melanocyte.

Electronmicroscopy has produced evidence to support this concept. Although the dense material seen in electronmicrographs may not be identical with melanin, it seems quite safe to consider that the changes seen after incubation with dopa may be due to *in vitro* melanization.

In order to separate the less melanosomes from more intensely melanized melanosomes, density-gradient tubes were prepared with sucrose solution (concentration 2.5 *M*—1.5 *M*) and the large-granule suspension isolated from Harding-Passey melanoma. The typical appearance of the tube after centrifugation in a horizontal rotor at 103,000 $\times g$ for 1 hour is quite similar to that shown in Fig. 1B: a narrow, brownish-gray, relatively tightly packed band is visible at the top of the gradient and a relatively clear zone lies between this

layer and the brown or black suspension which fills almost the entire bottom half of the tube. The fractions containing melanosomes of low density had two or three times more activity than the fraction containing melanosomes of high density. Electronmicrographs of these fractions did not, however, show any significant differences in degree of melanization of melanosomes isolated from the upper fractions (low density) and the lower fractions (high density).

SUMMARY

1. Melanosomes, the distinctive, enzymically active particles which are the site of melanin formation, have been separated from B-16 and Harding-Passey mouse melanoma and from retinal pigment-epithelium of chick embryos by density-gradient centrifugation.

2. The color and tyrosinase activity per milligram of protein-nitrogen of melanosomes isolated from these tissues have been compared. Melanosomes isolated from B-16 mouse melanoma showed greater optical density and less tyrosinase activity than those of Harding-Passey melanoma. Melanosomes isolated from the retinal pigment-epithelium of the 10-day-old chick embryo showed less optical density and higher tyrosinase activity than those isolated from the retinal pigment-epithelium of the 17-day-old chick embryo.

3. Melanosomes isolated from Harding-Passey mouse melanoma were melanized *in vitro* by incubation with dopa. The reaction velocity of tyrosinase in melanosomes which had been thus melanized *in vitro* was significantly decreased. There was an inverse linear relationship between the concentration of dopa in the incubation mixture and the reaction velocity of tyrosinase.

4. Under the electronmicroscope, melanosomes which had been melanized *in vitro* had an intensely electron-dense appearance which suggested that their surface had been covered chemically and mechanically by dopa-melanin which had been produced during incubation.

5. Experimental results suggest that as successive layers of melano-protein accumulate

on the melanosome, the active centers of tyrosinase are blocked and that therefore there exists a reciprocal relationship between degree of melanization and level of measurable tyrosinase within the melanosome.

The authors wish to thank Dr. M. S. C. Birbeck of the Chester Beatty Research Institute of London, for taking the electronmicrographs and for his valuable suggestions. They also want to express their appreciation of the help given by Drs. H. Blaschko and R. Baker of Oxford University during the later phases of this investigation.

This investigation was supported in part by the Damon Runyon Cancer Fund and by Research Grant #8196-2 from the United States Public Health Service.

REFERENCES

- (1) Lerner, A. B., and Fitzpatrick, T. B., *Physiol. Rev.*, **91**, 30 (1953)
- (2) Mason, H. S., in "Pigment Cell Biology" edited by M. Gordon, Academic Press, Inc., New York, p. 563 (1959)
- (3) Fitzpatrick, T. B., and Kukita, A., in "Pigment Cell Biology" edited by M. Gordon, Academic Press, Inc., New York, p. 489 (1959)
- (4) Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H., *J. Biol. Chem.*, **178**, 185 (1949)
- (5) Herrman, H., and Boss, M. B., *J. Cell. & Comp. Physiol.*, **26**, 131 (1945)
- (6) Seiji, M., and Fitzpatrick, T. B., *J. Invest. Dermat.*, in press.
- (7) a) Wellings, S. R., and Siegel, B. V., *J. Natl. Cancer Institute*, **24**, 437 (1960)
b) Wellings, S. R., and Siegel, B. V., *J. Ultrastructure Research*, **3**, 147 (1959)
- (8) Wissenfels, N., *Z. Zellforsch. u. mikroskop. Anat.*, **45**, 60 (1956)
- (9) Charles, A., and Ingram, J. T., *J. Biophys. Biochem. Cytol.*, **6**, 41 (1959)
- (10) Drochmans, P., *J. Biophys. Biochem. Cytol.*, **8**, 165 Vol. III (1960)
- (11) Birbeck, M. S. C., and Barnicot, N. A., in "Pigment Cell Biology" edited by M. Gordon, Academic Press, Inc., New York, p. 549 (1959)
- (12) Anderson, N. G., in "Physical Techniques in Biological Research" edited by Gerald Oster and Arthur W. Pollister, Academic Press, Inc., New York, Vol. **III** (1956)
- (13) De Duve, C., Berthet, J., and Beaufay, H., *Prog. in Biophys. & Biophys. Chem.*, **9**, 325 (1959)
- (14) Miyamoto, M., and Fitzpatrick, T. B., *Science*, **179**, 199 (1957)
- (15) Nelson, J. M., and Dawson, C. R., in "Advances in Enzymology" Interscience Publishers, Inc., New York, Vol. **4**, p. 99 (1944)
- (16) Ludwig, B. J., and Nelson, J. M., *J. Am. Chem. Soc.*, **61**, 2601 (1939)
- (17) Hogeboom, G. H., and Adams, M. H., *J. Biol. Chem.*, **145**, 273 (1942)

Immunochemical Studies on Catalase

I. Assay of Catalase in Erythrocytes and Liver

By TOKUHIKO HIGASHI*, MICHIKO YAGI and HIDEMATSU HIRAI

(From the Department of Biochemistry, Faculty of Medicine,
University of Tokyo, Tokyo)

(Received for publication, March 31, 1961)

The amount of catalase in erythrocytes, liver or other biological materials has been determined mostly on the basis of its enzymatic activity. Since many investigators have indicated the probable existence of inhibitory or stimulating substances for the enzymatic activity of catalase, such a determination is apparently easily disturbed and a careful consideration must be given so that it yields a precise value of the level of this enzyme.

The immunochemical assay by means of the quantitative precipitin reaction has been studied by Deutsch and Seabara (1) and is recognized to have the advantage of estimating the actual concentration of the enzyme protein, although involving the possibility of cross reaction with other proteins closely related to catalase.

It is a well known fact that a remarkable decrease in liver catalase is demonstrated in tumor-bearing animals, and hormones have been reported to increase the activity of catalase in liver, but the question whether these are merely changes in activity or are accompanied by an alteration in the amount of actual enzyme protein remains unsolved. As Deutsch *et al.* (1) suggested, the combination of these two kinds of assay method should provide us with a clue to answer this question, and also it may offer more advanced information on the situation of catalase in living tissues. Furthermore, it will be a useful tool for the approach to clarify the metabolism of this enzyme including the

biosynthesis of this specific protein molecule.

By using rabbit antibody against crystalline catalase from human erythrocytes, the present authors have investigated the level of catalase in human erythrocytes, rat erythrocytes and rat liver, with the simultaneous measurements of the enzymatic activity as well. This paper deals with the results, in details, obtained from such a study, an outline of which has been already published (2).

EXPERIMENTAL

Catalase as Antigen—From the hemolysates of human erythrocytes, catalase was highly purified in its crystalline form by the method of Herbert and Pinsent (3). The preparation was pure electrophoretically and ultracentrifugically.

Preparation of Anti-Catalase Serum—Rabbits were inoculated with this purified catalase suspended in Freund's adjuvant solution (4). Only a single injection of 10 mg. of catalase per rabbit was found enough to produce a fairly potent antiserum in 4 to 6 weeks. The antisera collected from immunized animals were pooled, inactivated at 56°C for 30 minutes, followed by lyophilization, and the resulting dried sera were dissolved in distilled water at the desired concentration just prior to use.

Immunochemical Assay of Catalase—Employing an excess amount of anti-catalase solution above described, the level of catalase in a given preparation was determined by the quantitative precipitin reaction (5). The specific precipitates were assayed by the phenol reagent method after dissolving with an alkali solution. The procedures were the same as those reported elsewhere by one of the authors (6).

Activity Measurements of Catalase—The enzymatic activity was measured titrimetrically according to the method of Bonnichsen *et al.* (7).

* The present address: c/o The Mary Imogene Bassett Hospital, Cooperstown, New York, U. S. A.

RESULTS

Calibration Curve—By the use of increasing amounts of purified crystalline catalase the calibration curve for its immunochemical assay has been established (Fig. 1).

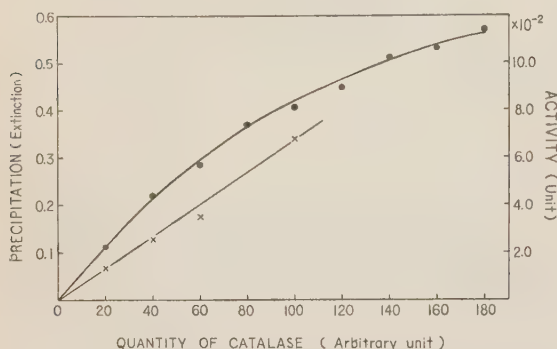


FIG. 1. A calibration curve for the immunochemical assay of catalase.*

—●—; precipitin reaction,
—×—; enzymatic activity.

Relationship between Precipitation and Activity

—The assay of the specific precipitate suspended in 3 M NaCl revealed that only 30 to 60 per cent of the original enzymatic activity was recovered, in agreement with Deutsch *et al.* (1). These values might be understood by consideration of the poor solubility of the complex and the inactivation of catalase during the period of the precipitin reaction. Tria (8) and also Campbell *et al.* (9) showed that the mixture of catalase and its antibody retained almost complete enzymatic activity.

Since the enzymatic activity of catalase is inhibited by the presence of cyanide or cysteine, the precipitin reaction was investigated under these conditions. Even in the presence of inhibitors in a concentration sufficient to suppress the action of catalase completely, the precipitation was scarcely

* Throughout this paper the amount of catalase was expressed in an arbitrary scale of units, and the following abbreviations were used: PU, units obtained by the immunochemical assay; AU, units calculated from the activity measurements. In the case of crystalline catalase used as the standard, PU and AU are exactly the same.

affected.

Therefore, it seems likely that the active site on a molecule of catalase which participates in the combination with its antibody may be different from the prosthetic group responsible for its enzymatic function.

Assay of Catalase in Human Erythrocytes—A hemolysate with water of human erythrocytes, after centrifuging at $14,000 \times g$ for 15 minutes, was submitted to the immunochemical determination of the amount of catalase as well as to the assay of its enzymatic activity. As indicated in Fig. 2, the level of catalase estimated immunochemically (PU) showed good correspondence with that calculated from the enzymatic activity.

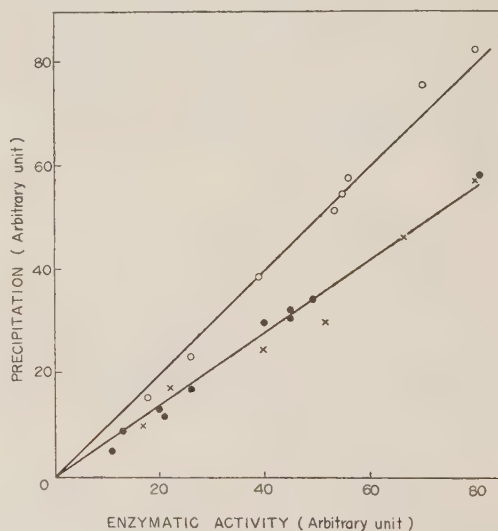


FIG. 2. The relationship between PU and AU in human and rat erythrocytes.

○; human, ●; normal rats,
×; tumor-bearing rats.

Estimation of Catalase in Rat Erythrocytes—

The rabbit antibody against catalase of human erythrocytes was found to give a considerable cross reaction with catalase of other species of animal except the rabbit. In hemolysates of rat erythrocytes, PU as determined by the use of the above calibration curve (Fig. 1) was constantly 70 per cent of AU as shown in Fig. 2. Instead of a cross reaction with a reduced extent of precipitation, it is possible, though not likely, to assume that rat erythrocyte catalase has a

higher enzymatic activity than that of human, or that a specific activator is present in rat hemolysates.

As studied so far, erythrocytes of tumor-bearing rats (ascites hepatoma, AH 49) showed no differences from those of normal animals. In all cases, a supernatant separated from the specific precipitate by centrifugation contained no detectable activity of catalase.

PU/AU Ratio in Rat Liver Homogenates—Table I shows the values of PU and AU in several preparations of rat liver homogenates from normal and tumor-bearing rats. Liver homogenates were made with 4 volumes of distilled water and assayed after being centri-

fuged at $14,000\times g$ for 15 minutes.

It appears that the ratio of PU to AU in liver homogenates is rather different from liver to liver, ranging from about 1:1 over 3:1.

Treatment of Liver Homogenates with Deoxycholate—Adams and Burgess (10) reported that mouse liver homogenates, when treated with a detergent, showed a rise in the activity of catalase which was attributed to a solubilization of the catalase involved in the large particulate fractions. They termed the catalase in liver homogenates before and after this treatment EPC* and *total catalase* respectively. Using this technique, treatment of rat liver homogenate** with sodium deoxycholate at a final concentration of 0.5 per cent in tris-hydroxymethylaminomethane buffer (pH 8.0) was investigated by the immunochemical assay as well as by the activity measurements. The data are summarized in Table II. Except for one case of tumor-bearing animal, PU/AU ratios were about the same both in the supernatant and the total catalase.

In another experiment, the supernatant fraction after centrifugation at $14,000\times g$ for 15 minutes did not exhibit any increase in catalase activity (PU or AU) by the treatment with a detergent.

PU and AU after Incubation of Liver Slices—After incubation in sucrose-phosphate buffer at 37°C for varying periods of time, rat liver slices were homogenized and determination

TABLE I
PU and AU in Rat Liver Homogenates

	PU	AU	PU/AU
Normal rat	27	18.5	1.46
	110	90	1.22
	197	101.5	1.94
	a) { 16	17	0.94
	37	34	1.09
	77	74	1.03
	b) { 49.5	14	3.54
	94	27	3.50
	145	42.5	3.42
	208	74.5	2.80
Tumor-bearing rat	165	89	1.85
	218	99	2.20

a) and b) The same preparation was examined at various dilutions.

TABLE II
Solubilization of Catalase with Deoxycholate

Expt. No.	I		II		III		IV	
Catalase unit	PU	AU	PU	AU	PU	AU	PU	AU
Supernatant catalase	26.5	25.5	62	37.5	20	19.5	213	89
	(1.03)		(1.65)		(1.03)		(2.49)	
Total catalase	58	53	148	102	66	61.5	344	198
	(1.10)		(1.45)		(1.08)		(1.74)	
Supernatant/total (%)	46	48	42	36	31	32	62	47

Figures in parentheses indicate PU/AU ratio in each preparation. I, II and III; normal rats, IV; a tumor-bearing rat.

* EPC means "extraparticulate cytoplasmic". Instead of this term, "Supernatant" was used in this article.

** 0.35 M sucrose-phosphate buffer solution (pH 7.6) was used.

TABLE III

Ratio of PU/AU and Supernatant/Total Catalase during Incubation

Incubation time (hrs.)		0	1	2	3
PU/AU	Supernatant catalase	1.46	1.22	1.08	1.14
	Total catalase	0.83	0.96	1.09	1.12
$\frac{\text{Supernatant catalase}}{\text{Total catalase}} \%$	AU	38.0	26.3	34.0	36.8
	PU	39.0	34.5	35.6	36.3

of PU and AU were made for both the total and the supernatant catalase. The results obtained are indicated in Fig. 3 and Table III.

It appears that PU/AU in the total catalase increased steadily with time while, on the contrary, the PU/AU of the supernatant catalase gradually decreased, and that some changes occurred after 1 hour incubation.

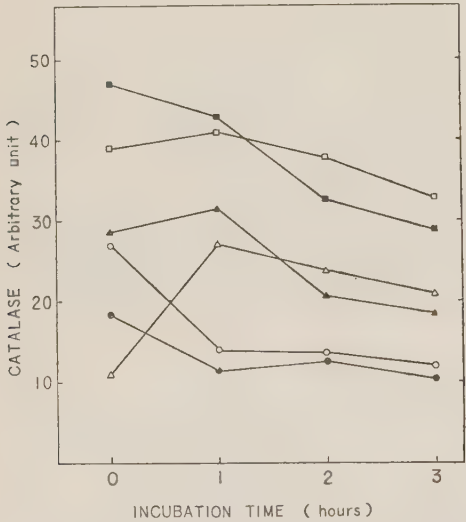


FIG. 3. Changes of PU and AU in the fractions of catalase during the incubation of rat liver slices.

Total {AU (■) PU (□)}
Supernatant {AU (●) PU (○)}
Particulate {AU (▲) PU (△)}

Examination of Ascites Tumor Cells—Rat ascites tumor cells were collected and ex-

TABLE IV

Assay of Catalase in Ascites Tumor Cells

Experimental No.	I	II	III
Activity (AU)	0	8.5	0
Precipitation (PU)	22	17	10

Ascites tumor cells were homogenized with 6 to 8 volumes of water and centrifuged. 0.2 to 1.0 ml. of the supernatant solution was used for the assay of the activity, and 2.0 ml. was used for the determination by the precipitin reaction.

tracted with water. As demonstrated in Table IV, a certain measurable precipitation took place on adding anti-catalase solution to these extracts, while practically no enzyme activity could be detected.

DISCUSSION

Although immunological cross reactions among the catalase of several species of animal has already been shown qualitatively (8, 9), the present study has shown quantitatively this reaction between human erythrocyte catalase and that of rat. A purified preparation of beef liver catalase also was found to react with antiserum against human erythrocyte catalase. This fact suggests another advantage in enabling one to select different species of animal, so that the one species might be suitable for obtaining a purified preparation of catalase as an antigen, and the other convenient for the desired experimental purposes. Moreover, it offers

the possibility of investigating the relationship between species specificity and the chemical nature of this enzyme.

In the assay of hemolysates PU is linearly proportional to AU.

Assuming that rat liver catalase is identical with that in erythrocytes (*cf.* (1, 10)), its PU/AU ratio might be expected to be 0.70. It is quite interesting that many findings from this study indicate a variable discrepancy between PU and AU in the given preparations of rat liver and tumor cell extracts, and therefore, suggest the existence of a protein(s) which possesses no enzymatic activity of catalase but is precipitable by the antiserum against catalase.

Adams and Burgess (11) have demonstrated a remarkable increase in the activity of mouse liver catalase after treatment with Triton X100. It will be recognized from the similar experiment using deoxycholate (Table II) that such an increase may involve, more or less, a rise in the actual concentration of the enzyme. With regard to the percentage of supernatant to total catalase, it is very likely that the difference in the conditions for homogenization results in the different values. Previous workers reported variable results of 30 per cent (11), 50 per cent (12) and 80 per cent (13), respectively. Based on experiments employing polyvinylpyrrolidone- or albumin-sucrose solutions for homogenization, Greenfield and Price (14) concluded that most, if not all, of the catalase of the rat liver cell is in mitochondria and is rapidly released into sucrose solutions. Thomson and Klipfel (15) found 20 per cent of the catalase in the supernatant fraction of rat liver with use of polyvinylpyrrolidone. The distribution of liver catalase into subcellular fractions seems worthy of study by means of the immunochemical assay.

Adams *et al.* (16, 17) have incubated mouse liver slices in a medium of phosphate buffer and demonstrated an intracellular redistribution of catalase in terms of enzymatic activity; the supernatant catalase increases with time due to the release from

larger granules, while the level of the total catalase is maintained constant. In this report, from the view point of a preliminary approach to the biosynthesis of this enzyme protein, the same kind of experiment has been traced with the immunochemical assay in addition to activity measurements. As shown above, a few different findings from those of the previous authors have been found as well as other additional evidence. If it is supposed that the immunological precipitates of catalase may be contaminated with other protein compounds which are related to catalase metabolism, these data suggest many interesting possibilities for research in this field.

SUMMARY

By means of an immunochemical assay as well as enzyme activity measurements, catalase in human erythrocytes, rat erythrocytes and liver has been investigated.

In erythrocytes, the level of catalase determined immunochemically was proportional to that estimated from its activity, but in preparations from rat liver and ascites tumor cells, considerable discrepancies were seen between these two values.

Based on the results obtained, the authors have suggested the existence of protein(s) exhibiting no enzymatic activity but reacting immunologically as catalase.

The authors wish to express their gratitude to Prof. N. Shimazono for his continuous encouragements and interest during the course of this work, and to Dr. K. Warabioka of the Cancer Institute, Tokyo, for his kind gift of tumor-bearing animals. Their thanks are also due to Dr. T. Peters, Jr. of the Mary Imogene Bassett Hospital, Cooperstown, New York, who gave helpful advices and discussions.

REFERENCES

- (1) Deutsch, H. F., and Seabara, A., *J. Biol. Chem.*, **214**, 455 (1955)
- (2) Higashi, T., Yagi, M., and Hirai, H., *J. Biochem.*, **47**, 696 (1960)
- (3) Herbert, H., and Pinsent, J., *Biochem. J.*, **43**, 203 (1948)
- (4) Freund, J., and McDermott, K., *Proc. Soc.*

- Exptl. Biol. Med.*, **49**, 548 (1942)
- (5) Heidelberger, M., and Kendall, F. E., *J. Exptl. Med.*, **65**, 697 (1935)
- (6) Higashi, T., and Hasegawa, F., *J. Biochem.*, **48**, 71 (1960)
- (7) Bonnichsen, R. K., Chance, B., and Theorell, H., *Acta Chem. Scand.*, **1**, 685 (1947)
- (8) Tria, E., *J. Biol. Chem.*, **129**, 377 (1939)
- (9) Campbell, D. H., and Fourt, L., *J. Biol. Chem.*, **129**, 385 (1939)
- (10) Bonnichsen, R. K., *Arch. Biochem.*, **12**, 83 (1947)
- (11) Adams, D. H., and Burgess, E. A., *Brit. J. Cancer*, **11**, 310 (1957)
- (12) Ludewig, S., and Chanutin, A., *Arch. Biochem.*, **29**, 441 (1950)
- (13) von Euler, H., and Heller, L., *Z. Krebsforsch.*, **56**, 393 (1949)
- (14) Greenfield, R. E., and Price, V. E., *J. Biol. Chem.*, **220**, 607 (1956)
- (15) Thomson, J. F., and Klipfel, F. J., *Cancer Research*, **18**, 229 (1958)
- (16) Adams, D. H., and Berry, M. E., *Biochem. J.*, **64**, 492 (1956)
- (17) Adams, D. H., Burgess, F. A., *Biochem. J.*, **71**, 340 (1959)

Gas-chromatographic Analysis of the Fatty Acid Composition of Human Fatty Liver

By YOSHIYATA TAKAHASHI and KAY TANAKA

(From the Third Department of Internal Medicine, Faculty of Medicine,
University of Tokyo, Tokyo)

(Received for publication, February 28, 1961)

Although a great deal of work has already been done on clinical and experimental fatty liver, details of the pathogenesis is not yet elucidated. Until recently it was not possible to determine the fatty acid composition of liver tissue accurately because of the limitation in analytical methods. Introduction of gas-chromatography for lipid analysis in 1956 (1) provided an extremely useful method to study this problem. We have therefore undertaken a gas-chromatographic analysis of fatty acid composition of the human fatty liver and mesenteric adipose tissue.

MATERIALS AND METHODS

Materials—Liver specimens from three cases of fatty liver showing numerous fat droplets in the liver cells were used. The first case was that of acute cyanide poisoning for the purpose of suicide, and the second was a six years old child with cyclic vomiting. The third specimen was obtained from a case of fatty cirrhosis with history of alcoholism, who died in an accident.

As a control eight liver specimens from autopsy materials without visible pathological changes were used.

Methods—The fresh liver tissue (5 to 10 g.), after addition of a small amount of hydroquinone, was homogenized in 200 ml. of chloroform-methanol (2:1) with a Waring blender. The extract was evaporated to dryness *in vacuo* and hydrolyzed in 8% methanolic KOH at 60°C for four hours. After evaporation of the major part of methanol *in vacuo* the solution was shaken with petroleum ether (b.p. 40–60°C) three times. The solution was then acidified and shaken with ethyl ether. After the ether layer was evaporated to dryness, HCl-methanol and anhydrous aluminum sulfate were added and refluxed for two hours. Methanol was evaporated *in vacuo* and the methyl

esters were dissolved in *n*-hexane. The hexane layer was washed with water, then with dilute sodium bicarbonate solution, and concentrated under a stream of nitrogen.

Gas-chromatographic analysis was carried out at 200°C in a brass tube of 2.5 m. long and 4 mm. internal diameter. Reoplex 400 (2) was used as stationary phase and helium as carrier gas. Detector was of the thermal conductivity type. Flow rate of about 100 ml./min. was used. For the quantitative determination synthetic arachidic acid was used as an internal standard.

Column chromatography of lipids was carried out essentially as described by Barron and Hanahan (3). Silicic acid (Mallinckrodt, 100 mesh) was activated at 110°C over night and kept in an air tight bottle. When use, 20 g. of the silicic acid was dusted into a chromatographic tube of 15 mm. diameter. Approximately 40 ml. of acetone-ether (1:1), 20 ml. of ethyl ether and 500 ml. of petroleum ether were then passed through successively. After the lipid materials dissolved in a small amount of hexane were loaded, 350 ml. of 15% benzene in *n*-hexane, 300 ml. of peroxide free ethyl ether and 400 ml. of methanol were applied. Fractions of 50 ml. each were collected, the small aliquots of which were tested for cholesterol, glyceride and phospholipid on each chromatography by using the Liebermann-Burchard reaction, Van Handel Zilversmit's method (4) and Allen's method (5) respectively. It was found that ferric chloride reaction could not be used for the determination of cholesterol in the liver tissue because of the lack of specificity. There was at least one component which was positive on ferric chloride reaction but negative on Liebermann-Burchard reaction.

RESULTS

In both liver and adipose tissue, fatty acids found constantly were myristic, palmitic, palmitoleic, stearic, oleic and linoleic acid.

In some occasion there were observed several peaks showing the same retention time as those of myristoleic, pentadecanoic, heptadecanoic, linolenic and arachidic acids. However, since the percentages of these peaks were so small and the identifications of these substances were not complete, it was decided that they would better be omitted from the calculations. Although eicosatrienoic acids were frequently found, their separation were not so perfect enough to permit the accurate determinations that they were included in one group as C20 unsaturated acids (C20F).

The Fatty Acid Camposition of the Liver

Total Lipid—In the non-fatty liver where no distinct pathological change was found the most abundant fatty acid was palmitic acid accounting for 34.0 per cent of the total fatty acid. The next abundant was oleic acid (24.0 per cent) followed by linoleic, and

stearic acid. On the other hand, in fatty liver the most abundant fatty acid was oleic acid reaching 40.2 per cent. There were also striking increases of palmitoleic acid and myristic acid and distinct decrease of stearic acid portion (Table I).

As compared with the fatty acid composition in the control group the analysis of fatty acid revealed an about twofold increase in the oleic acid portion and an about four times increase in palmitoleic acid, which is also a monoethenoid acid. On the other hand the percentages of stearic acid were decreased to about one eighth of that in control group. Octadecadienoic acid which was proved to be mainly linoleic acid by oxidative degradation (6) decreased to about one half of the control.

Cholesterol Ester Fraction—The most abundant fatty acid in cholesterol ester of the control liver was oleic acid followed by palmitic

TABLE I
Fatty Acid Composition of the Fatty Liver, Expressed as Percentages the Total

Fatty Liver			In percentages of total						
	Age Sex	Diagnosis	Myris- tic	Palmi- tic	Palmi- toleic	Stearic	Oleic	Linoleic	C20F
20193	53♂	Cyanide Poisoning	4.4	26.6	14.3	0.6	42.1	11.1	trace
20210	35♂	Fatty Cirrhosis	6.2	30.2	13.6	1.6	36.9	10.5	trace
20909	5♀	Cyclic Vomiting	2.5	29.4	13.8	3.7	41.6	9.0	trace
Mean			4.4	28.7	13.9	2.0	40.2	10.2	—
Control									
21158	18♂	Intrahepatic Aneurism	0.6	39.1	2.9	9.1	25.2	19.9	3.1
20910	62♂	Mitral Insufficiency	1.7	22.6	4.6	13.7	31.4	23.4	2.7
21080	62♂	Gastric Cancer	0.6	31.2	3.4	14.5	28.3	19.3	2.7
21124	74♂	//	0.4	33.1	2.2	18.4	20.3	18.6	6.6
20911	48♂	Aortic Insufficiency	1.3	33.6	4.4	20.1	21.4	18.8	trace
21136	41♀	Encephalomyelitis	0.2	43.3	3.6	21.2	17.5	14.4	trace
21171	34♂	Chron. Myel. Leukemia	0.5	33.9	1.6	26.6	17.5	15.2	3.5
21053	36♂	Trauma	0.5	35.2	8.2	12.8	30.1	13.1	trace
Mean			0.7	34.0	3.9	17.1	24.0	17.8	2.3

TABLE II

*Fatty Acid Composition of Cholesterol Ester in the Liver,
Expressed as Percentages of the Total*

(1) Cholesterol Ester

			Fatty Liver			In percentages of total			
Age Sex		Diagnosis	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	C20F
20193	53♂	Cyanide Poisoning	2.5	29.2	11.0	7.0	25.0	13.2	3.9
20210	36♂	Fatty Cirrhosis	7.1	28.2	21.8	0.6	26.6	12.3	trace
20909	5♀	Cyclic Vomiting	2.5	29.4	13.8	3.7	41.6	9.0	trace
Mean			4.0	28.9	15.5	3.8	31.1	11.5	1.3
Control									
21124	74♂	Gastric Cancer	1.9	31.9	5.3	14.2	34.5	12.4	trace
20910	62♂	Mitral Insufficiency	trace	26.6	6.7	trace	50.0	16.7	trace
21053	36♂	Trauma	1.3	18.1	5.8	5.8	41.8	19.3	7.7
Mean			1.1	25.5	5.9	6.6	42.1	16.1	2.6

TABLE III

*The Fatty Acid Composition of the Neutral Fat Fraction of the Liver,
Expressed as Percentages of the Total*

(2) Neutral Fat

			Fatty Liver			In percentages of total			
Age Sex		Diagnosis	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	C20F
20193	53♂	Cyanide Poisoning	5.2	33.1	13.0	2.8	35.3	8.5	0
20210	36♂	Fatty Cirrhosis	7.3	29.4	14.5	3.2	36.7	7.9	0
20909	5♀	Cyclic Vomiting	2.4	29.6	14.2	2.1	43.9	7.9	0
Mean			5.0	30.7	14.5	2.7	38.6	8.1	0
Control									
21124	74♂	Gastric Cancer	1.0	39.1	3.8	10.5	30.5	15.1	0
20910	62♂	Mitral Insufficiency	0.9	17.0	5.2	13.6	39.9	18.2	5.2
21053	36♂	Trauma	2.4	31.4	10.3	3.2	37.3	14.3	1.2
Mean			1.4	29.2	6.4	9.1	35.9	15.9	2.1

acid. In fatty liver, on the other hand, there was a definite increase of palmitoleic acid and some decrease of linoleic acid (Table II).

The Neutral Fat Fraction—In both fatty liver and the control the amount of non-

esterified fatty acid was so small as compared with that of glyceride that no attempt was undertaken to separate the two fractions. The fatty acid pattern obtained on this fraction may presumably be taken as that of glyceride (Table III).

TABLE IV

The Fatty Acid Composition of Phospholipid Fraction of the Liver,
Expressed as Percentages of the Total

(3) Phospholipid

			Fatty Liver			In percentages of total			
Age Sex	Diagnosis		Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	C20F
20193	53♂	Cyanide Poisoning	1.0	37.4	5.0	17.2	22.2	12.3	2.7
20210	36♂	Fatty Cirrhosis	2.1	34.1	7.6	10.9	30.5	14.5	trace
20909	5♀	Cyclic Vomiting	0.2	26.7	6.1	21.7	22.6	13.7	8.7
Mean			1.1	32.7	6.2	16.6	25.1	13.5	3.8
Control									
21124	74♂	Gastric Cancer	0.7	37.2	1.2	19.3	16.4	15.6	8.9
20910	62♂	Mitral Insufficiency	trace	32.1	1.7	26.5	12.3	19.8	7.6
21053	36♂	Trauma	trace	33.2	6.2	25.0	20.2	15.4	trace
Mean			0.2	34.2	3.0	23.6	16.3	16.9	8.3

In this fraction the most abundant fatty acid was oleic acid in both fatty liver and control. In fatty liver there were twice as much palmitoleic acid and half as much linoleic acid as in the control. There were also noted an increase of myristic acid in fatty liver. The percentage of stearic acid was also decreased.

Phospholipid Fraction—The phospholipid fraction was characterized by the most abundant presence of palmitic acid and much higher percentage of stearic acid than in other lipid fractions. With regard to the fatty acid compositions, myristic, palmitoleic and oleic acid were increased in fatty liver as compared with the control. On the contrary, stearic and linoleic acid were decreased in fatty liver (Table IV).

Summarizing these results it can be concluded that in fatty liver there are increase in the percentages of palmitoleic, oleic and myristic acid and decrease in the percentages of stearic and linoleic acid in all the three lipid fractions.

However it should be noted that these changes in the fatty acid patterns of three lipid fractions in fatty liver are not so striking as the changes in total fatty acid (Figs.

1, 2). While fatty acid composition of the control liver resembles that of their phospholipid fractions, the fatty acid patterns of the fatty liver simulates that of either cholesterol ester or neutral fat fraction. This

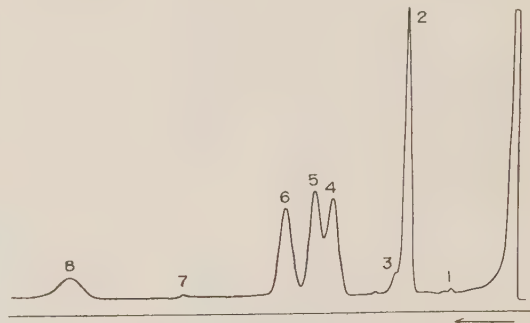


FIG. 1. Gas-chromatographic pattern of the total fatty acid in the control liver (Case No. 21124); (1) myristic, (2) palmitic, (3) palmitoleic, (4) stearic, (5) oleic, (6) linoleic, (7) arachidic, (8) C20 unsaturated acid.

would indicate that the major lipid fraction of the fatty liver consists of either cholesterol ester or neutral fat. Quantitation of the absolute amount of the fatty acid in these fractions is therefore required.

TABLE V

The Amount of the Individual Fatty Acid in the Liver

In mg./100 g. fresh liver tissue.

Fatty Liver							mg./100 g. tissue.			
	Age Sex	Diagnosis	Total Fatty Acids	Mristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	C20F
20210	36♂	Fatty Cirrhosis	309×10 ²	1930	9460	4220	500	11520	3290	trace
20809	5♀	Cyclic Vomiting	228×10 ²	560	6720	3160	840	9480	2080	trace
Control										
21158	18♂	Intrahepatic Aneurysm	8800	48	3360	274	825	2245	1810	242
20910	62♂	Mitral Insufficiency	3320	56	768	155	465	1063	796	19
21080	62♂	Gastric Concer	3540	20	1105	120	512	1000	683	96
20911	74♂	Aortic Insufficiency	1420	19	476	67	286	305	266	trace
21171	48♂	Chr. Myel. Leucemia	1380	6	472	21	362	238	212	69
21053	36♂	Trauma	2650	11	935	218	339	800	350	trace
Mean			3520	27	1186	143	465	942	686	71

The Absolute Amount of Individual Fatty Acid in the Liver

Total Lipid—There was a remarkable increase of total fatty acid in fatty liver

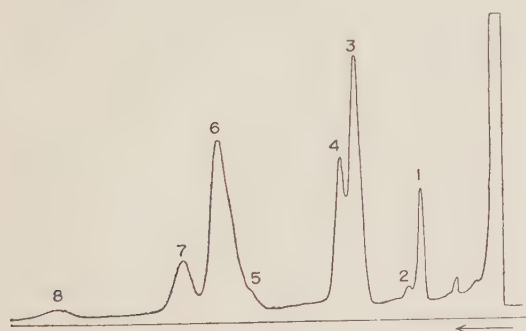


FIG. 2. The gas-chromatographic pattern of the total fatty acid in the fatty liver (case No. 20210); (1) myristic, (2) myristoleic, (3) palmitic, (4) palmitoleic, (5) stearic, (6) oleic, (7) linoleic, (8) arachidic acid.

(about eight times as much as in the control). All the individual fatty acid except stearic acid showed definite increase. Interestingly linoleic acid increased to about four times as

much as that of control (Table V).

Cholesterol Ester Fraction—In a case of fatty cirrhosis a remarkable increase of the total fatty acid in this fraction was noted. Linoleic acid was also increased. In another case of fatty liver there was a slight increase of fatty acid as compared with the control (Table VI).

Phospholipid Fraction—An increase of all the fatty acid including linoleic acid was observed in fatty liver.

Neutral Fat Fraction—The most pronounced increase of all the fatty acids was found in this fraction and linoleic acid was not an exception.

These results clearly demonstrate that the change of fatty acid pattern of the total lipid in fatty liver is secondary to the relative increase of neutral fat fraction.

The Fatty Acid Composition of the Adipose Tissue

The fatty acid pattern of the mesenteric adipose tissue was also characteristic (Fig. 3, Table VII). The highest percentage was found in the oleic acid fraction and the

TABLE VI

The Amount of the Individual Fatty Acid in the Three Major Lipid Fractions of the Liver

		Cholesterol Ester				In mg. per 100 g. of liver			
	Diagnosis	Total Fatty Acids	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	C20F
20210	Fatty Cirrhosis	1710	126	500	386	11	471	219	trace
20909	Cyclic Vomiting	80	0	3	1	3	8	1	2
	Control	11	trace	2	1	1	4	2	1

Phospholipid									
20210	Fatty Cirrhosis	2510	54	857	192	276	767	367	0
20909	Cyclic Vomiting	1060	2	283	65	230	240	144	91
	Control	693	trace	230	43	173	140	107	trace

Neutral Fat									
20210	Fatty Cirrhosis	267 × 10 ²	1750	8093	3642	205	10282	2704	0
20909	Cyclic Vomiting	232 × 10 ²	563	6860	3300	488	10170	1840	0
	Control	1867	45	585	193	59	696	267	22

TABLE VII

The Fatty Acid Composition of Adipose Tissue, Expressed as Percentage of the Total

		Control				In percentages of total			
Age	Sex	Diagnosis	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	C20F
21136	41 ♀	Encephalomyelitis	4.4	29.4	7.9	1.6	42.8	12.9	0
20910	62 ♂	Mitral Insufficiency	4.9	25.4	9.8	2.7	42.1	13.9	1.4
20911	48 ♂	Aortic Insufficiency	3.2	23.2	5.8	3.4	41.8	22.6	0
21291	70 ♀	Cancer of G. Bladder	1.8	22.8	6.7	1.8	48.6	16.5	1.8
Mean			3.6	25.2	7.6	2.4	43.8	16.5	0.8

Fatty Liver Patient									
20909	5 ♀	Cyclic Vomiting	3.1	34.4	10.1	3.1	43.9	3.8	1.6

lowest in the stearic acid. There seems to be a slight difference in fatty acid pattern of the adipose tissue between cases of fatty liver and control.

In control cases some difference was observed in the fatty acid pattern of the

neutral fat between liver and mesenteric adipose tissue. The percentage of stearic acid tended to be higher in the liver. On the other hand, only minor difference was found in fatty acid composition of neutral fat fraction between fatty liver tissue and adipose

tissue. This result would be quite interesting in view of the mechanism of fatty liver development.

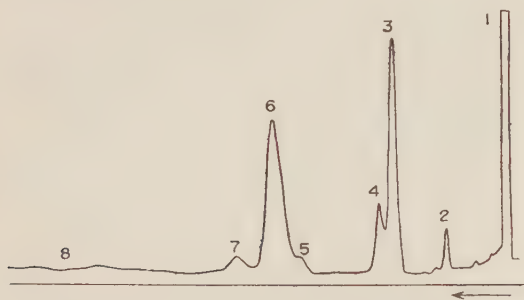


FIG. 3. The fatty acid pattern of the mesenteric adipose tissue of a case of fatty liver; (1) solvent, (2) myristic acid, (3) palmitic acid, (4) palmitoleic acid, (5) stearic acid, (6) oleic acid, (7) linoleic acid, (8) C20 unsaturated acid.

DISCUSSION

The fatty acid pattern of the total lipid of control liver was quite similar to that of the phospholipid fraction, indicating the phospholipid to be the main fraction in the control liver. On the other hand the fatty acid pattern of the total lipid in the fatty liver was very close to those of the cholesterol ester and neutral fat fraction. The actual determinations of the total fatty acid in cholesterol ester, neutral fat and phospholipid fractions demonstrated that the most abundant lipid fraction was neutral fat fraction in fatty liver, thus proving that the fatty acid pattern of the fatty liver was actually representing that of the enormously increased neutral fat. This conclusion is in good agreement with the results of the determination of neutral fat in the human fatty liver (7).

As regard the origin of this accumulated fat in the liver there are still controversy of opinions. While many workers believe in the extrahepatic origin, some insist on the synthesis *in situ*. In this respect it is noteworthy that there is a definite increase of linoleic acid in fatty liver. There are ample evidences that linoleic acid is not appreciably synthesized in human tissue. Lipsky *et al.* (8) found only slight radioactivity in the lino-

leic acid fraction of the human plasma lipids after injection of C^{14} -acetate. Recently James *et al.* (9) found C^{14} -activity in linoleic acid fraction of red blood cells incubated with C^{14} -acetate *in vitro*. However, since the radioactivity found by these workers apparently is not so high as observed in other fatty acid fractions, the possibility of artefact cannot be entirely excluded. More recently Fulco and Mead (10) suggested an alternative explanation. Basing on the opinion that linoleic acid is not synthesized in human body, the present result showing a definite increase of linoleic acid in fatty liver should be interpreted as indicating that there were inflow of linoleic acid into the liver during the development of fatty liver. Since the transport of linoleic acid alone without being accompanied by other fatty acid moiety has never been reported, other fatty acids must have entered the liver simultaneously. Although the origin of these fatty acid is obscure at present it is quite probable that they were transported from the adipose tissue because of the close similarity of the fatty acid patterns between the neutral fat fractions of the liver and the adipose tissue.

In non-fatty liver the fatty acid pattern of the neutral fat fraction of the liver differs from that of the adipose tissue, showing the higher percentage of stearic acid. One might well consider that the fatty acid pattern of neutral fat fraction becomes quite similar to that of adipose tissue presumably as a consequence of the import of a large amount of neutral fat into the liver from adipose tissue and its insufficient disposal by liver cells.

SUMMARY

1. The fatty acid compositions of fatty liver, control liver, and adipose tissue were analyzed by gas-chromatography.

2. The fatty acid pattern of total lipids in fatty liver simulates that of the neutral fat fraction, thus indicating the major lipid fraction to be neutral fat.

3. There were four fold increase of linoleic acid content in fatty liver.

4. A close similarity of fatty acid patterns

were found between the neutral fat fractions of the liver and the adipose tissue in the case of fatty liver. From these results an extra-hepatic origin of the neutral fat within fatty liver are suggested.

Thanks are due to Prof. Dr. S. Okinaka for his constant encouragement of this work.

REFERENCES

- (1) James, A. T., and Martin, A. J. P., *Biochem. J.*, **63**, 144 (1956)
- (2) Orr, C. H., and Callen, J. E., *J. Am. Chem. Soc.*, **80**, 249 (1958)
- (3) Barron, E. J., and Hanahan, D. J., *J. Biol. Chem.*, **231**, 493 (1958); Hanahan, D. J., "Lipide Che-

mistry" John Wiley and Sons, Inc. New York p. 38, (1960)

- (4) Van Handel, E., and Zilversmit, D. B., *J. Lab. Clin. Med.*, **50**, 152 (1957)
- (5) Allen, R. J. L., *Biochem. J.*, **34**, 858 (1940)
- (6) Tanaka, K., and Takahashi, Y., "The 33rd General Meeting of Japan. Biochem. Soc." (1960)
- (7) Kobayakawa, K., *Trans. Soc. Path. Jap.*, **48**, 1518 (1959)
- (8) Lipsky, S. R., Haavik, A., Hopper, G. L., Mc Divin, R. H., and Mossberg, B. M., *J. Clin. Invest.*, **36**, 233 (1957)
- (9) James, A. T., Lovelock, J. E., and Webb, J. P. W., *Biochem. J.*, **73**, 106 (1959)
- (10) Fulco, A. J., and Mead, J. F., *J. Biol. Chem.*, **235**, 3379 (1960)

Conversion of Prothrombin into Thrombin

I. DEAE-Cellulose Chromatography of Prothrombin and Isolation of a Prothrombin Derivative

By TOSHIO ASADA, YUZABURO MASAKI, KOICHI KITAHARA, RYUICHI NAGAYAMA, TOSHIYUKI HATASHITA and ISAMU YANAGISAWA

(From the Department of Biochemistry, School of Medicine, Toho University, Tokyo)

(Received for publication, March 10, 1961)

The conversion of prothrombin into thrombin is one of the most important reactions included in a complicated mechanism of blood coagulation, and, as a model of conversion from "zymogen" into enzyme, it would be the most interesting one, of which much yet remains to be done in comparison with detailed studies on the conversion of proteases by Neurath *et al.* (1).

As the pioneer in this field, Seegers and his school first purified bovine prothrombin (2) in homogeneous state through ultracentrifugation and their studies concerning the conversion of prothrombin into thrombin indicated the presence of a similar autocatalytic mechanism, both in non-physiological and physiological activation as in the protease activation. Therefore the theory presented by Seegers (3) seems to be inconsistent with the classical theory of blood coagulation and even to the modern concepts of the blood thromboplastin generation test by Biggs and MacFarlane (4). Recently, Alexander (5) purified prothrombin in another way and, though his sample was not so homogenous, proconvertin content in prothrombin sample was very poor and showed a striking two-stage unreactivity to thromboplastin, Ac-globulin

and calcium. In this meaning, Seegers' sample would be more contaminated with other clotting factors, because his sample has some reactivity to thromboplastin and calcium.

Further purification of Seegers' prothrombin was carried out by Miller (6) using the ion exchanger, Amberlite IR-50, column chromatography. Though Seegers' preparation of prothrombin seemed to be uniform on test with ultracentrifuge analysis and its specific activity on the chromatogram was found to be the same as before, some impurity of the sample was found in the chromatographic pattern.

However, chromatography of proteins by means of synthetic resins presents a certain difficulty. Recently, Peterson and Sober (7) introduced cellulose ion exchangers with the aim of overcoming these difficulties. The authors made efforts for further purification of bovine prothrombin prepared according to the method of Seegers and also of thrombin converted from purified prothrombin in different activation state, by means of cellulose ion exchangers (8-10).

In the presented series of work, appropriate conditions for DEAE-cellulose chromatography of prothrombin were determined and this method was found to be available for the study on purification process, including thrombin. A prothrombin derivative or a presumable intermediate protein between prothrombin and thrombin was isolated by this method from the prothrombin samples.

Following abbreviations were used; DEAE for diethylaminoethyl. TAME for tosylarginine-methyl-ester. CTN for carbobenzoxy-tyrosinyl-*p*-nitrophenol. Ac-globulin for factor V. Proconvertin for factor VII in plasma. SPCA (Serum prothrombin conversion accelerator) for factor VII in serum. PTC (Plasma thromboplastin component) or Christmas factor for factor IX.

EXPERIMENTAL MATERIALS AND METHODS

Prothrombin—Bovine prothrombin was purified according to the method of Ware and Seegers (2). The total activity yield and specific activity during the purification process are shown in Table I. The original process concerning fractionation with ammonium sulfate was partially modified to 45–66% saturation.

TABLE I

Yield of Prothrombin Units and Specific Activity during the Purification Procedure (Bovine plasma, 3 liters)

	Total units in NIH unit (IOWA unit)	Specific activity
Precipitate of diluted plasma at pH 5.1–5.2	2.7×10^5 (6.2×10^5)	209(480)/mg. N
Decomposition of Mg (OH) ₂ cream by CO ₂ gas	1.5×10^5 (3.4×10^5)	488(1,122)/mg. N
Fractionation with ammonium sulfate (0.45–0.66%)	0.5×10^5 (1.1×10^5)	2,928(6,734)/mg. Tyrosine
Supernatant at pH 5.4 Isoelectric precip. at pH 4.6	3×10^4 (7.0×10^4)	8,750(22,125)/mg. Tyrosine

Determination of Prothrombin Units—a) Modified Brinkhous' two-stage method (11): The use of acacia gum in the original method was substituted with 0.02 M calcium chloride solution. For use as a substrate, bovine fibrinogen was purified according to the method of Ware, Guest, and Seegers (12), and its clottability was between 90 and 95 per cent. Rabbit brain thromboplastin prepared according to the method of Quick (13) and partially purified bovine lung thromboplastin according to the method of Chargaff *et al.* (14) were used as tissue thromboplastin. Standardization of unit was performed by means of Thrombin (Park Davis) in NIH unit, which was found to have a single peak of activity in DEAE-cellulose chromatography and equivalent to TAME units of Sherry and Troll (15).

b) The two-stage method of Ware and Seegers (16): The activated thrombin unit was calculated from the Table indicating the relationship between clotting time and correlation factors (IOWA unit).

Determination of Thrombin Units—Clotting units was determined similar as in prothrombin. Determination of TAME hydrolyzing unit was carried out according

to the method of Sherry and Troll (15). The hydrolyzing activity of thrombin for CTN was determined by an improved method (19), which was modified from Axelrod *et al.* (17, 18).

DEAE-Cellulose Chromatography—DEAE-cellulose was prepared from cellulose powder (Toyo Roshi) according to the method of Peterson and Sober (7), whose nitrogen equivalent per milligram was between 1.0 and 0.6. The size of column was about 18×150 mm. Gradient method was used at a low temperature for the chromatography and the volume of a mixture was usually 200 ml. being changed in proportion to protein content.

EXPERIMENTAL RESULTS

Preliminary Batch Test for Chromatography—

For the batch test, crude prothrombin was used, which was prepared omitting the final isoelectric precipitation according to the method of Ware and Seegers. As shown in Fig. 1, the result of batch test on a small scale indicates the relationship of dissociation between DEAE-cellulose and prothrombin in varying pH and salt concentrations.

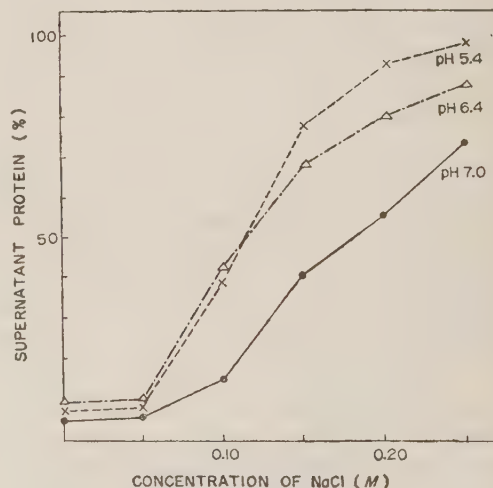


FIG. 1. Dissociation between DEAE-cellulose and prothrombin preparation.

DEAE-Cellulose Chromatography of Prothrombin—The result of preliminary tests indicated that the adsorption of prothrombin on the column would be effected in 0.005 M phosphate buffer (pH 7.0) and that elution of prothrombin would be carried out in 0.05 M phosphate buffer (pH 5.4)—0.4 M sodium

chloride in the use of gradient method. Fig. 2 indicates that these presumed conditions were suitable for the gradient method because percentage recovery of prothrombin activity was about 100 per cent.

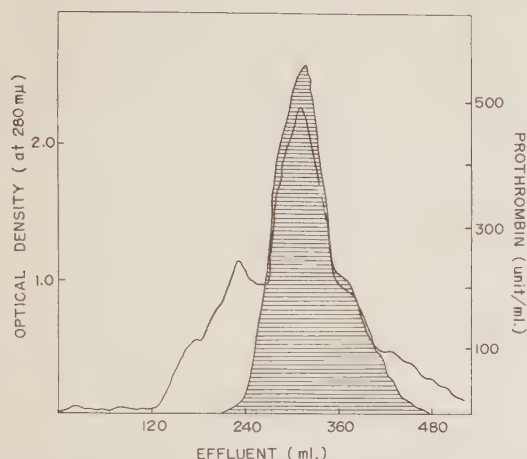


Fig. 2. DEAE-cellulose chromatography of crude prothrombin.

Prothrombin (fractionation with ammonium sulfate) containing 50,000 NIH units (115,000 IOWA units) was adsorbed to the column of DEAE-cellulose (20 mm. \times 200 mm.) at pH 7.0 using 0.005*M* phosphate buffer. Gradient elution method was employed with 0.05*M* phosphate buffer (pH 5.4). Constant volume mixture was 200 ml.

—: Optical density, : Prothrombin

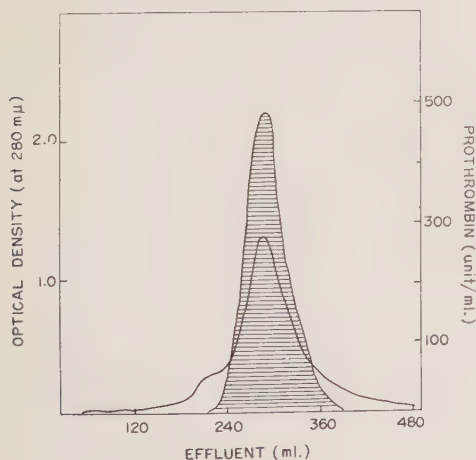


Fig. 3. DEAE-cellulose chromatography of purified prothrombin. Prothrombin (final step) containing 30,000 NIH units (70,000 IOWA units) and no thrombin was chromatographed by the conditions same as in Fig. 2.

As shown in Fig. 3, purified prothrombin in the final step still contains a considerable amount of non-prothrombin protein. The main fraction of activity was collected, dialyzed, and then lyophilized. However, specific activity of this DEAE-cellulose-treated prothrombin was 5680 units/mg. tyrosine, showing partial lowering of the activity, which is presumably due to long dialysis.

This DEAE-cellulose-treated prothrombin behaved as a single protein when tested by ultracentrifuge analysis (Fig. 4) and paper



Fig. 4. Ultracentrifuge analysis DEAE-cellulose-treated prothrombin 1% phosphate solution pH 7.0 $\mu=0.1$ 59,730 r.p.m. Time interval, 16 minutes.

electrophoresis (Fig. 5). Rechromatography of this sample by DEAE-cellulose, however,

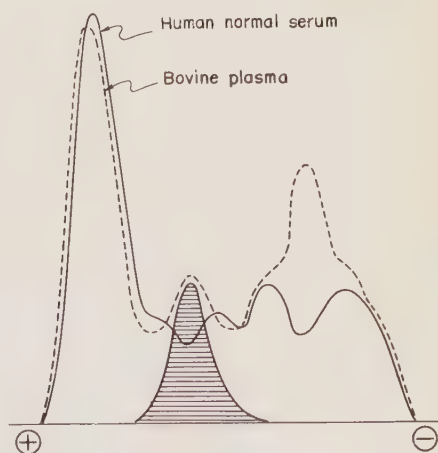


Fig. 5. Paper electrophoresis of DEAE-cellulose-treated prothrombin.

indicated the presence of a trace of non-prothrombin protein (Fig. 6).

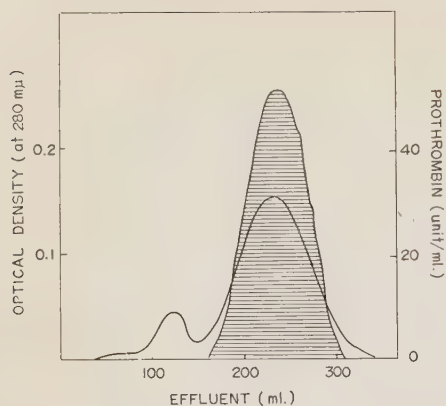


FIG. 6. Rechromatography of DEAE-cellulose-treated prothrombin mixture (volume, 100ml.).

The Reactivity of DEAE-Cellulose-treated Prothrombin to Calcium and Tissue Thromboplastin System—Seegers' preparation of prothrombin still contained a small amount of Ac-globulin (3) and, concerning the presence of proconvertin (plasma factor VII or plasma type of SPCA) Seegers postulated that "auto prothrombin-I" coming from prothrombin during the autocatalytic activation would have a similar effect as factor VII, and the activity of factor VII would be due to a prothrombin derivative. According to Alexander, plasma filtered through a Seitz filter contains a smaller amount of proconvertin than the decreased content of prothrombin (4) and, therefore, Alexander's prothrombin must have striking unreactivity.

As shown in Fig. 7, DEAE-cellulose-treated prothrombin still retained slight reactivity and thrombin formation occurred very slowly. The addition of Ac-globulin obviously accelerated the rate of thrombin formation, while addition of SPCA had no effect on the rate of thrombin formation. Consequently, this chromatographic procedure would be effective against the contamination of Ac-globulin, but the nature of prothrombin remains unchanged concerning the proconvertin or factor VII.

Isolation of a Prothrombin Derivative—During these series of chromatographic observation, an interesting derivative of prothrombin was found. As shown in Fig. 8, prothrombin

sample was contaminated with a trace of thrombin, presumably activated slowly during

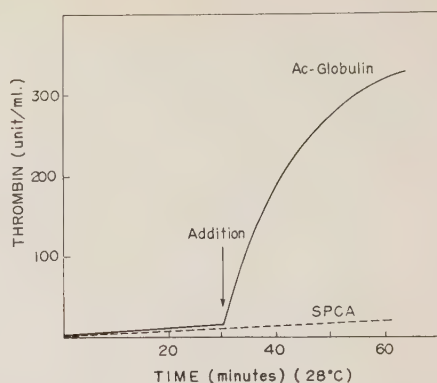


FIG. 7. Activation of DEAE-cellulose-treated prothrombin in calcium and tissue thromboplastin system.

The reaction system was similar to that in the two-stage method. DEAE-cellulose-treated prothrombin was activated very slowly with calcium and thromboplastin. After 30 minutes, a small amount of plasma Ac-globulin or serum prothrombin conversion accelerator (SPCA) was added to the mixture. Plasma Ac-globulin was a crude sample, which was used in the two-stage method according to the method of Brinkhous. SPCA was purified according to the method of Alexander.

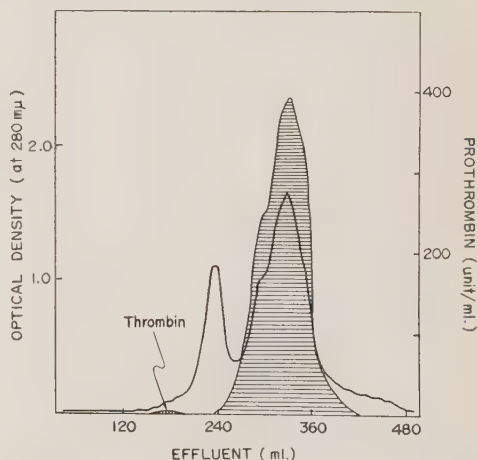


FIG. 8. DEAE-cellulose chromatography of purified prothrombin containing a trace of thrombin.

Prothrombin, 30,000 NIH units (70,000 IOWA units) and thrombin; 30 units before chromatography.

the fractionation with ammonium sulfate, a fairly sharp peak of protein having no prothrombin activity and also no thrombin activity appeared in front of the prothrombin peak and located between thrombin and prothrombin.

TABLE II

Conversion of Prothrombin Derivative into Thrombin

The sample isolated from the chromatogram shown in Fig. 8 was dialyzed and then lyophilized.

Sample activated by 25% sodium citrate solution	Thrombin produced	
	0 hour	after 10 hours
1% Derivative	50 U/ml.	2,020 U/ml.
1% Prothrombin	50 U/ml.	5,080 U/ml.

50 U/ml. thrombin was added as a catalyst.

This fraction was not activated by calcium and thromboplastin system, but was easily converted into thrombin by the addition of a catalytic amount of thrombin in 25% citrate solution. The specific activity of the thrombin formed was about one-half of that of prothrombin.

Moreover, Fig. 9 indicates an interesting pattern of chromatography, which suggests a mode of degradation process of prothrombin molecule in the autocatalytic activation. This sample contained 50,000 units of prothrombin before chromatography, decreased its prothrombin content to only 300 units after the chromatography, while the thrombin content increased from 300 to 500 units. It was found that the second-eluted protein peak had a nature similar to that of prothrombin derivative shown in Fig. 8.

Application of DEAE-Cellulose Chromatography of Prothrombin to the Analysis of Physiological Activation—DEAE-cellulose chromatography of prothrombin has been proved to be suitable not only for prothrombin, but for activated thrombin as shown in Figs. 8 and 9. Both the thrombin chromatography introduced by Rassmussen (20) and prothrombin chromatography by Miller (6) could not eluate each activity in a single chromatography, and

the simultaneous chromatography for both prothrombin and thrombin would be the characteristic of DEAE-cellulose.

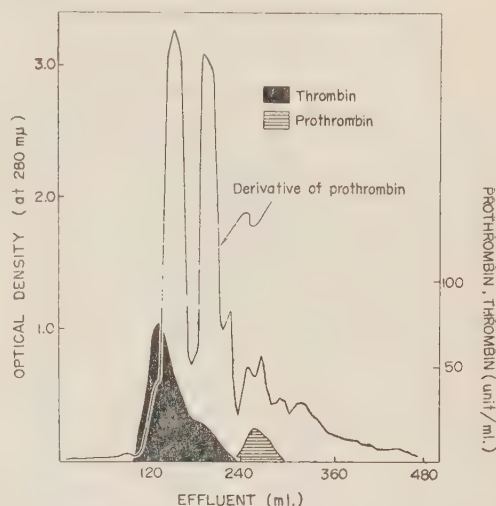


FIG. 9. DEAE-cellulose chromatography of prothrombin containing considerable amount of thrombin.

Before chromatography: Prothrombin about 50,000 NIH units (115,000 IOWA units) and thrombin 300 units. After the chromatography, there were found about 5,000 units of thrombin and 300 units of prothrombin. The second peak of protein was easily converted to thrombin in 25% sodium citrate solution, while the first peak was unchanged by the same condition.

Application of this technique to the study on the mechanism of physiological activation was attempted. As shown in Table III, purified prothrombin according to Ware and Seegers was activated by the addition of calcium and bovine lung thromboplastin, which was quantitatively centrifuged down by an ultracentrifuge after activation process. Table III also shows simultaneously three different activities of thrombin concerning fibrinogen, TAME, and CTN during the physiological activation of prothrombin. After centrifuging down lung thromboplastin at 40,000 r.p.m. for 25 minutes, clear solution was dialyzed in the cold and submitted to DEAE-cellulose chromatography. Fig. 10 shows its chromatographic pattern, in which trace activity of prothrombin was detectable

and only thrombin activity was indicated as a single peak of activity. Remarkable changes

Further research by this means is now in progress.

TABLE III

Activation of Prothrombin by Calcium Bovine Lung Thromboplastin

Reaction system: Total volume 15.0 ml.; which contained 9.0 ml. of 1.5% prothrombin (Ware, Seegers), 3.0 ml. of 0.05*M* imidazole buffer (pH 7.2), 0.3 ml. of bovine lung thromboplastin suspension, 2.1 ml. of 0.85% NaCl, and 0.6 ml. of 0.1*M* CaCl₂ (final 0.004*M*). Room temperature (17°C).

Formed Thrombin Units			
Incub. time (min.)	Clotting Unit	TAMe Unit	CTN Unit
5	304	304	170
15	825	788	228
25	1,260	1,560	336
35	1,440	1,680	673
45	1,890	1,980	720
60	1,980	2,205	—

The mixture was centrifuged at 40,000 r.p.m., and dialyzed against 0.005*M* phosphate buffer (pH 7.0) for 12 hours. The chromatographic pattern of this sample is shown in Fig. 10.

were found in the protein peak in comparison with the above described patterns of DEAE-cellulose chromatography.

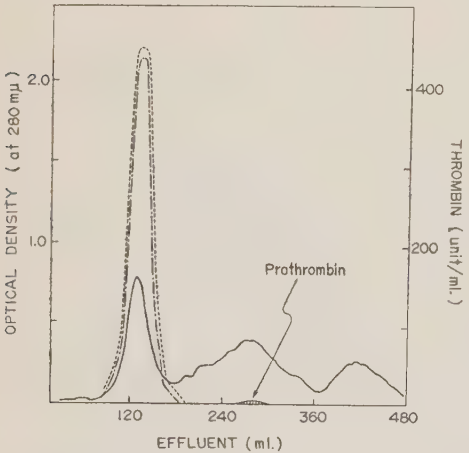


Fig. 10. DEAE-cellulose chromatography of prothrombin after activation by calcium and lung thromboplastin.

- Clotting Unit (NIH),
- TAME Unit (Sherry).

DISCUSSION

In the chromatographic purification of prothrombin, a problem which consistently arises is the possibility of separating prothrombin from the coagulation factors, namely proconvertin and PTC (Christmas Factor), etc., which are known to have a similar behavior in their adsorption to the inorganic precipitate, barium sulfate, magnesium hydroxide, and aluminium hydroxide.

The results obtained by the present authors suggested that even chromatographic procedure with DEAE-cellulose could not separate prothrombin from factor VII or proconvertin. Presumably these adsorptive clotting factors would be very similar in molecular weight and protein structure to prothrombin molecule.

The DEAE-cellulose-treated prothrombin was examined in detail concerning its chemical composition, protein structure, and physico-chemical properties. These data will be soon published.

A problem concerning the isolation of a prothrombin derivative has been presented. The concept of prothrombin derivative was first introduced by Seegers, *et al.* (3) while studying the disappearance of prothrombin activity and the increased thrombin activity at the initial stage of citrate activation, because during the first one hour, larger part of prothrombin activity is lost and no corresponding units of thrombin are detectable. They therefore concluded that the lost prothrombin would be presumably converted into a prothrombin derivative, which would be insensitive to the thromboplastin calcium system, namely, the two-stage reagent, and retained the properties as the precursor of thrombin. In this meaning, the concept of prothrombin derivative is a hypothetical protein, mainly discussing the difference of the total sum of prothrombin units disappeared and thrombin units newly activated.

The prothrombin derivative isolated in the present work was insensitive to the two-

stage reagent and easily converted into thrombin by autocatalytic activation in citrate solution. These properties seem to be similar to a hypothetical prothrombin derivative, but, its further identification must be carried out in detail. It seems rather interesting that the location of the prothrombin derivative was midway between the prothrombin peak and thrombin peak on the chromatographic pattern, and the electrophoretic behavior during the citrate activation was just the same as that of prothrombin (unpublished data).

The application of DEAE-cellulose chromatography suggests that further research in the conversion process of prothrombin by means of this chromatography would promising in this field, because in the simultaneous elution by a single chromatography, prothrombin, thrombin, and even a prothrombin derivative, as well as other similar proteins would be isolated separately.

SUMMARY

1. After the batch test, chromatography with DEAE-cellulose was carried out as follows. Prothrombin purified according to the method of Ware and Seegers was adsorbed on the column with 0.005 *M* phosphate buffer (pH 7.0) and eluted with 0.05 *M* phosphate buffer (pH 5.4)-0.4 *M* sodium chloride by the gradient method.

2. Some properties of DEAE-cellulose-treated prothrombin were discussed.

3. In the chromatographic pattern of purified prothrombin contaminated with a trace of thrombin, it was found that a sharp protein peak having neither prothrombin nor thrombin unit was a prothrombin derivative, which easily converted into thrombin by autocatalytic activation in a citrate solution.

4. DEAE-cellulose chromatography was applied to the study of the conversion mech-

anism by physiological activation of prothrombin into thrombin.

REFERENCES

- (1) Neurath, H. E., *Advances in Protein Chem.*, **XII**, 319 (1957)
- (2) Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, **174**, 565 (1948)
- (3) Seegers, W. H., 'Proc. IVth Intl. Congr. Biochem. Vienna', **X**, 18 (1958)
- (4) Alexander, B., 'Proc. IVth Intl. Congr. Biochem. Vienna', **X**, 37 (1958)
- (5) Biggs, R., and MacFarlane, R. G., "Human Blood Coagulation", Oxford (1957)
- (6) Miller, K. D., *J. Biol. Chem.*, **231**, 987 (1958)
- (7) Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **78**, 751 (1958)
- (8) Asada, T., Yanagisawa, I., Kitahara, K., and Nagayama, R., 'Proc. XIth Enzyme Chem.', 135, Kyoto (1954)
- (9) Asada, T., Isamu, Y., Koichi, K., Masaki, K., Hatashita, T., and Amano, H., 'Proc. XIIth Enzyme Chem.', 92, Sendai (1960)
- (10) Asada, T., Yanagisawa, I., Kitahara, K., Masaki, Y., and Nagayama, R., 'Proc. VIIIth Intl. Congr. Hematol.', 241 (1960)
- (11) Brinkhous, et al., "The Techniques of the Blood Coagulation", ed. Tocantins, New York (1955)
- (12) Ware, A. G., Guest, M. M., and Seegers, W. H., *Arch. Biochem.*, **13**, 331 (1947)
- (13) Quick, A. J., *Am. J. Clin. Pathol.*, **15**, 560 (1945)
- (14) Chargaff, E., Moore, D. H., and Bendich, A., *J. Biol. Chem.*, **145**, 593 (1942)
- (15) Sherry, S., Troll, W., *J. Biol. Chem.*, **208**, 95 (1954)
- (16) Ware, A. G., Seegers, W. H., *Am. J. Clin. Pathol.*, **19**, 471 (1949)
- (17) Martin, C. T., Golubow, T., and Axelrod, A. E., *J. Biol. Chem.*, **234**, 294, (1959)
- (18) Martin, C. T., Golubow, T., and Axelrod, A. E., *J. Biol. Chem.*, **234**, 1718 (1959)
- (19) Aoki, E., *J. Japan. Biochem. Soc.*, **33**, in press
- (20) Rassmussen, P. S., *Biochim. et Biophys. Acta*, **16**, 157 (1955)

Effect of Estradiol, Nucleotides and Bicarbonate on the Metabolism of Glucose and Malonate*

By NORIMASA HOSOYA** and NOBUKO KAWADA

(From the Department of Biochemistry, Tokyo Women's Medical College, Tokyo)

(Received for publication. March 8, 1961)

Malonate was a poor inhibitor of endogenous respiration of human placental slices and decreased the oxygen consumption below the level of endogenous respiration when glucose was added as a substrate. In a previous paper, it was reported that malonate could exert a regulatory effect on glucose metabolism in human placental slices and in addition malonate, itself, was metabolized (2). Further investigations concerning this phenomenon were carried out. In the present paper the additions of 17 β -estradiol, adenosine triphosphate, diphosphopyridine nucleotide and sodium bicarbonate to placental slices in the presence of glucose and malonate were investigated. The effect of these substances on the metabolic rate are reported using uniformly labeled glucose and malonate-2- C^{14} .

METHODS

The tissue slice and homogenate of human placenta (gestational age 13–20 weeks) were obtained according to the method in our previous report (2).

In the tissue slice experiments, free hand slices of young placenta weighing about 200 mg. were placed

in conventional Warburg vessels in 3.0 ml. of a medium containing, per milliliter, 40 μ moles K^+ , 80 μ moles Na^+ , 10 μ moles Mg^{++} , 100 μ moles Cl^- , and 40 μ moles of phosphate, with an initial pH of 6.9. The medium contained the C^{14} -labeled substrate***; uniformly labeled glucose or/and potassium malonate-2- C^{14} . The solution containing the C^{14} -labeled substrate was placed in the side arm of the Warburg flask and tipped into the main reaction chamber after the vessels were gassed with oxygen for 7 minutes. In the incubation medium, the final concentration of glucose was 10 μ moles per ml. and malonate was 15 μ moles per ml. (about 7×10^3 c.p.m. per μ mole for each substrate).

After one hour incubation at 37°C the slices of tissue were removed, blotted dry on filter paper, and weighed. The extraction of total lipids from slices for C^{14} analysis was carried out by the method of Folch *et al.* (3) and the isolation of glycogen from slices by the method of Villee *et al.* (4). The respiratory carbon dioxide was trapped in alkali placed in the center well (0.2 ml. of 5% carbonate-free solution of sodium hydroxide), and converted to barium carbonate for C^{14} analysis by the method of Villee *et al.* (4). Analysis for content of C^{14} was made with a windowless, proportional flow counter.

In the homogenate experiments each vessel contained 3.0 ml. of the same reaction mixture as reported previously (2). The medium contained glucose, succinate or malonate as substrate. The final concentration of glucose and succinate was 10 μ moles per ml. and malonate was 15 μ moles per ml., unless stated to be otherwise. In those experiment in which uniformly labeled glucose and potassium malonate-2- C^{14} were used, the respiratory carbon dioxide was analysed by the same method as in the tissue slice experiment.

In all experiments, 17 β -estradiol in the form of an aqueous suspension was added to give a final concentration of $4 \times 10^{-6} M$ and sodium bicarbonate (pH 6.9 (checked by diluted HCl)) 10 μ moles was added in 3.0 ml. of reaction mixture. In the tissue slice experiment adenosine triphosphate (ATP) was added, 5 μ moles, and diphosphopyridine nucleotide (DPN), was added, 1.5 μ moles in 3.0 ml. of a medium.

* This report was presented at the 33rd meeting of the Japanese Biochemical Society, on November 1, 1960 in Tokyo (1). This work was supported in part by a grant for scientific research from the Ministry of Education.

** Present Address; Department of Biochemistry and Nutrition, the Division of Health Care and Nursing, Faculty of Medicine, the University of Tokyo, 120, Zoshigaya, Bunkyo, Tokyo.

*** Uniformly labeled glucose- C^{14} and calcium malonate-2- C^{14} were purchased from Daiichi Chemical Company, Tokyo. Calcium malonate-2- C^{14} was converted to potassium malonate-2- C^{14} by using an ion exchange resin.

RESULTS

Oxygen Consumption of Tissue Slice—Oxygen consumption of human placental slices in the presence of a mixture of glucose and malonate as a substrate is shown in Table I. In this case, oxygen uptake is decreased to about three fourth of the endogenous respiration.

TABLE I

Oxygen Consumption of Human Young Placental Slice

	Determination	QO ₂
No addition	40	3.02±0.24
Glucose, Malonate	40	2.25±0.19
Glucose, Malonate, Estradiol	10	3.29±0.32
Glucose, Malonate, ATP	8	3.50±0.28
Glucose, Malonate, DPN	8	3.46±0.27
Glucose, Malonate, NaHCO ₃	6	3.08±0.22

The values are expressed in QO₂ ± standard error of the mean. Glucose, 30 μ moles; malonate, 45 μ moles; estradiol, final concentration $4 \times 10^{-6}M$; ATP, 5 μ moles; DPN, 1.5 μ moles; NaHCO₃, 10 μ moles.

In previous communication we reported that this phenomenon indicated that malonate exert an inhibitory effect on the metabolism of glucose. A further experiments were carried out in the presence of 17 β -estradiol, ATP, DPN or sodium bicarbonate. The addition of sodium bicarbonate restored the oxygen consumption to endogenous levels, whereas ATP, DPN and estradiol resulted in oxygen uptake above endogenous levels. To elucidate this stimulation of the oxygen consumption, further experiment was carried out using uniformly labeled glucose and malonate-2-C¹⁴.

Carbon Dioxide Production of Tissue Slice—Conversion of isotopic carbon of the substrate to carbon dioxide in human placental slice is shown in Table II. Stimulation of carbon dioxide production by estradiol is observed in the tissue slice experiment. Carbon dioxide production from glucose-U-C¹⁴ in the presence of malonate with estradiol is almost the same as glucose-U-C¹⁴ alone. This evidence indi-

TABLE II

Conversion of Caabon of Substrate to Carbon Dioxide in Human Young Placental Slice

	Determination	Glucose-U-C ¹⁴		Malonate-2-C ¹⁴	
			Malonate		Glucose
No addition	6	0.106 ±0.013	0.038 ±0.006	0.004 ±0.001	0.005 ±0.001
Estradiol	5	0.142 ±0.009	0.111 ±0.011	0.102 ±0.018	0.092 ±0.011
ATP	4	0.075 ±0.006	0.046 ±0.005	0.140 ±0.020	0.078 ±0.007
DPN	4	0.212 ±0.026	0.115 ±0.016	0.204 ±0.011	0.173 ±0.011
NaHCO ₃	3	0.018 ±0.003	0.016 ±0.002	0.004 ±0.001	0.005 ±0.001

The values are expressed in μ moles per g. of wet weight of tissue per hour ± standard error of the mean.

cates that estradiol stimulates the rate of so-called isocitric dehydrogenase activity as shown by Vilee *et al.* (5). On the other hand, estradiol increased the carbon dioxide production from malonate-2-C¹⁴ to about twenty times that of the control.

The increase in the rate of carbon dioxide production by ATP is observed in the case of malonate-2-C¹⁴ used as a substrate. The addition of ATP may result in the stimulation in the rate of activation of malonate to malonyl-CoA in the first step during the course of malonate metabolism.

Stimulation of carbon dioxide production by DPN is observed in the tissue slice experiment. The addition of DPN may stimulate the rate of oxidation of glucose and malonate.

The addition of bicarbonate decreases the rate of carbon dioxide production as is observed when glucose-U-C¹⁴ is utilized as a substrate.

Glycogen Synthesis in Tissue Slice—Conversions of isotopic carbon of the substrate to glycogen in placental slice are shown in Table III. Stimulation of glycogen synthesis by estradiol is observed in the tissue slice experiment. Glycogen synthesis from glucose-U-C¹⁴ in the presence of malonate with estradiol results in a 8 fold stimulation.

The addition of ATP, DPN or bicarbo-

nate resulted in a decrease of glycogen synthesis from glucose-U-C¹⁴. Glycogen synthesis from glucose-U-C¹⁴ in the presence of malonate with bicarbonate is about five times that of the control.

TABLE III

Conversion of Carbon of Substrate to Glycogen in Human Young Placental Slice

	Determination	Glucose-U-C ¹⁴		Malonate-2-C ¹⁴	
			Malonate		Glucose
No addition	6	0.185 ±0.035	0.025 ±0.003	0.060 ±0.008	0.059 ±0.006
Estradiol	5	0.352 ±0.025	0.218 ±0.026	0.121 ±0.009	0.102 ±0.008
ATP	4	0.039 ±0.006	0.035 ±0.006	0.058 ±0.005	0.078 ±0.012
DPN	4	0.050 ±0.008	0.033 ±0.004	0.042 ±0.005	0.037 ±0.004
NaHCO ₃	3	0.099 ±0.007	0.121 ±0.009	0	0

The values are expressed in μ moles per g. of wet weight of tissue per hour \pm standard error of the mean.

TABLE IV

Conversion of Carbon of Substrate to Lipid in Human Young Placental Slice

	Determination	Glucose-U-C ¹⁴		Malonate-2-C ¹⁴	
			Malonate		Glucose
No addition	6	0.090 ±0.005	0.019 ±0.002	0.018 ±0.003	0.014 ±0.002
Estradiol	5	0.090 ±0.009	0.107 ±0.012	0.044 ±0.004	0.110 ±0.007
ATP	4	0.141 ±0.028	0.038 ±0.007	0.041 ±0.009	0.034 ±0.009
DPN	4	0.078 ±0.004	0.005 ±0.002	0.036 ±0.006	0.004 ±0.001
NaHCO ₃	3	0.031 ±0.004	0.012 ±0.002	0	0

The values are expressed in μ moles per g. of wet weight of tissue per hour \pm standard error of the mean.

Lipid Synthesis in Tissue Slice—Conversions of isotopic carbon of the substrate to total lipid in placental slice are shown in Table IV. Stimulation of total lipid synthesis by the addition of ATP is observed in the tissue

slice experiment.

Estradiol stimulates the rate of total lipid synthesis from a mixture of glucose and malonate. The total lipid synthesis from malonate-2-C¹⁴ is increased slightly with the addition of estradiol, ATP or DPN. These evidences indicate that malonate, adenine nucleotide and estradiol are activators in lipid synthesis. Concerning this result, Brady (6), Wakil (7) and Hosoya *et al.* (8) demonstrated that malonyl-CoA was an intermediate of fatty acid synthesis from acetate and Hosoya *et al.* (9) demonstrated that estradiol stimulated the fatty acid synthesis from acetate *in vitro*.

Effect of Estradiol on Respiration in Homogenates—Estradiol stimulates oxygen consumption, carbon dioxide production, glycogen synthesis and lipid synthesis in the tissue slice experiment. Using human placental homogenate, effect of estradiol on the oxygen consumption in the presence of glucose and malonate was investigated (Table V).

Estradiol stimulated the oxygen consumption of the homogenate without substrate or with glucose. Oxygen consumption in the presence of malonate or a mixture of glucose and malonate was not affected by estradiol.

On the other hand, effect of estradiol on conversion of isotopic carbon of the substrate to carbon dioxide was observed (Table VI). Estradiol increased the carbon dioxide production from glucose-U-C¹⁴ and malonate-2-C¹⁴. This evidence indicates that estradiol stimulates the rate of the citric acid cycle in the metabolism of glucose and the metabolism of malonate. Mueller *et al.* (10) reported

TABLE V

Effect of Estradiol on Oxygen Consumption in Human Young Placental Homogenate

	Determination	No substrate	Glucose	Malonate	Glucose Malonate
No addition	8	11.37 ± 0.82	5.72 ± 0.62	14.4 ± 0.98	15.97 ± 1.35
Estradiol	6	12.72 ± 0.93	11.40 ± 0.70	14.5 ± 0.85	15.97 ± 1.24

The values are expressed in QO₂ (N) \pm standard error of the mean.

TABLE VI

Effect of Estradiol on Conversion of Carbon of Substrate to Carbon Dioxide in Human Young Placental Homogenate

	Determination	Glucose-U-C ¹⁴		Malonate-2-C ¹⁴	
			Malonate		Glucose
No addition	8	1.64 ±0.066	1.78 ±0.130	1.92 ±0.089	1.64 ±0.110
Estradiol	4	1.83 ±0.072	1.95 ±0.061	3.81 ±0.250	2.34 ±0.126

The values are expressed in μ moles per g. of nitrogen of homogenate per hour \pm standard error of the mean.

that estradiol stimulated the level of amino acid activating enzymes in the rat uterus and Hosoya *et al.* (11) reported that estradiol stimulated the acetylation of sulfanyl amide in the pigeon liver acetone powder. From this evidences, estradiol might be considered to stimulate the activation of malonic acid to malonyl-CoA.

Effect of Bicarbonate on Oxygen Consumption in Homogenate—The effect of bicarbonate on the oxygen consumption was investigated further using placental homogenate (Table VII). Bicarbonate increased slightly the endogenous respiration and had no effect on the oxygen consumption in the presence of succinate. In the presence of malonate or a mixture of succinate and malonate as substrate, oxygen consumption was increased with bicarbonate nearly to that observed in the succinate alone.

TABLE VII

Oxygen Consumption of Human Young Placental Homogenate

Substrate	Determination	No addition	Succinate	Malonate	Succinate Malonate
NaHCO ₃					
No addition	10	11.39 ±0.663	20.92 ± 1.86	15.44 ± 1.09	16.09 ± 0.73
NaHCO ₃	4	12.45 ±1.25	20.87 ± 2.82	20.45 ± 3.75	19.48 ± 2.12

The values are expressed in $QO_2(N) \pm$ standard error of the mean. Succinate, 30 μ moles; malonate, 30 μ moles; sodium bicarbonate, 10 μ moles.

This evidence indicates that malonate and bicarbonate might produce the metabolite which had a higher oxygen consumption (β). The same effect of malonate and bicarbonate was observed in respiration of homogenate of ox retina by Burgess *et al.* (12).

DISCUSSION

Oxygen consumption of human placental slices in the presence of a mixture of glucose and malonate as a substrate was decreased to the degree of about three fourth of the endogenous respiration. The further addition of 17 β -estradiol, ATP, DPN or bicarbonate resulted in a recovery of oxygen consumption to endogenous levels. To elucidate this phenomenon, the experiment was carried out using uniformly labeled glucose and malonate-2-C¹⁴.

The addition of estradiol resulted in an increase in carbon dioxide production, glycogen synthesis and lipid synthesis from glucose and malonate. Glycogen and lipid synthesis from glucose in the presence of malonate and lipid synthesis from malonate in the presence of glucose were increased. Estradiol may stimulate the rate of the metabolism in placental slices. For this reason, oxygen consumption of tissue slice in the presence of a mixture of glucose and malonate would be increased.

On the other hand, increasing rate of carbon dioxide production from malonate is higher in the tissue slice than in the homogenate experiment. The oxygen consumption in the presence of malonate or a mixture of glucose and malonate in the homogenate experiment is on a higher level than without substrate and is not stimulated by estradiol. From these evidences, it may follow that estradiol can stimulate the rate of cell membrane permeability of malonate.

Carbon dioxide production from glucose was decreased by the addition of ATP whereas the production from malonate was increased in the tissue slice. Lipid synthesis from glucose and malonate was increased slightly by the addition of ATP. ATP, also, would stimulate the rate of permeability of malonate. The carbon dioxide production from glucose

and malonate was increased by the addition of DPN to the tissue slice. ATP and DPN do not pass through the cell membrane. Therefore, ATP and DPN would be decomposed, in part, outside of the cell. Constituents of the adenine nucleotides would reach into the cell, and ATP and DPN would be resynthesized inside of the cell to stimulate the oxygen consumption.

The addition of bicarbonate to tissue slice resulted in a decrease of carbon dioxide production, glycogen synthesis and lipid synthesis from glucose and malonate except in the case of the glycogen synthesis from glucose in the presence of malonate. Glucose and malonate themselves might not be utilized directly in the case of the addition of bicarbonate, therefore endogenous metabolic substances would be metabolized. The oxygen consumption in the homogenate experiment with malonate or a mixture of malonate and succinate as substrates was increased to the level of succinate by the addition of sodium bicarbonate. From this evidence it may be that malonate and bicarbonate could readily pass through the cell membrane and that any substrate could be synthesized in the cell. This substrate has the same level of oxygen consumption as the succinate. For this reason, oxygen consumption of a mixture of glucose and malonate would be increased by the addition of bicarbonate in tissue slice experiment.

SUMMARY

The metabolism of glucose and malonate were observed using human placental slices and homogenates.

1. The decrease in the rate of oxygen consumption of tissue slice in the presence of a mixture of glucose and malonate was restored nearly to that observed in the endogenous respiration by the addition of estradiol, ATP, DPN or bicarbonate.

2. Carbon dioxide production in tissue

slices was stimulated by estradiol, ATP and DPN with malonate as a substrate. In the homogenate, the level of carbon dioxide production from malonate with estradiol was higher than the glucose.

3. Glycogen synthesis from glucose in the presence of malonate was increased by the addition of estradiol and bicarbonate to the tissue slice.

4. Stimulation of lipid synthesis was observed with the addition of estradiol and ATP. Lipid synthesis from a mixture of glucose and malonate was increased by the addition of estradiol to the tissue slice.

5. In the homogenate, the oxygen consumption with a mixture of glucose and malonate was higher than without substrate. The oxygen consumption in the presence of this mixture was not stimulated by estradiol. By the addition of bicarbonate to malonate, oxygen consumption was increased nearly to that observed with succinate as a substrate.

REFERENCES

- (1) Hosoya, N., Kawada, N., and Matsumura, Y., *J. Japan. Biochem. Soc.*, **32**, 601 (1960)
- (2) Hosoya, N., Kawada, N., and Matsumura, Y., *J. Biochem.*, **48**, 262 (1960)
- (3) Folch, J., Lee, M., and Sloane Stanley, G. H., *J. Biol. Chem.*, **226**, 497 (1957)
- (4) Villet, C. A., and Hastings, A. B., *J. Biol. Chem.*, **179**, 675 (1949)
- (5) Villet, C. A., and Hagerman, D. D., *J. Biol. Chem.*, **233**, 1 (1958)
- (6) Brady, R. O., *Proc. Natl. Acad. Sci.*, **44**, 993 (1958)
- (7) Wakil, S. J., *J. Am. Chem. Soc.*, **80**, 6465 (1958)
- (8) Hosoya, N., Kawada, N., and Matsumura, Y., *Symposium on Enz. Chem. Japan*, **14**, 190 (1960)
- (9) Hosoya, N., Hagerman, D. D., and Villet, C. A., *Biochem. J.*, **76**, 297 (1960)
- (10) McCorquodale, D. J., and Mueller, G. C., *J. Biol. Chem.*, **232**, 31 (1958)
- (11) Hosoya, N., and Kawada, N., *J. Japan. Biochem. Soc.*, **31**, 734 (1959)
- (12) Ann Burgess, E., Kerly, M., and Rahman, M. A., *Biochem. J.*, **74**, 4 p (1960)

An Attempt to Demonstrate the Distribution of Trypsin Inhibitors in the Sera of Various Animals

By SHOJIRO NAKAMURA and TOSHIRO WAKEYAMA

(From the Institute for Medical Chemistry, Yamaguchi Medical School, Ube)

(Received for publication, March 6, 1961)

The first observation that there are two components of human serum which inhibit trypsin was made by Grob (1). He found the inhibitory activity to be predominantly associated with the α - and β -globulins of serum. Shulman (2, 3) confirmed that human serum contains two trypsin inhibitors. Later Jacobsson (4, 5) has demonstrated by the paper electrophoresis that two trypsin inhibitors are localized in α_1 - and α_2 -globulins. After some unsuccessful attempts by several workers (5-9), Wu and Laskowski (10) finally succeeded in crystallizing an acid-labile trypsin inhibitor from bovine blood plasma.

The distribution of trypsin inhibitors in the fractions of human serum proteins was studied rather precisely by Jacobsson (4, 5) by the following procedure: Serum was first separated into fractions by paper electrophoresis, each fraction was extracted separately, cutting the filter paper into sections, and the inhibitory activity of each extracted fraction was estimated by the use of trypsin and its substrate. Because of the complexity of the method, distribution of trypsin inhibitors in sera of animals other than humans has not yet been elucidated, although the first crystalline inhibitor was obtained from bovine plasma.

Recently Nakamura and his coworkers (11) have demonstrated by the two-dimensional crossing paper electrophoresis that there are two trypsin inhibitors in the extract of soy beans. By the same method, the distribution of trypsin inhibitors in the sera of various animal species has been demonstrated. The results will be reported in the present paper.

EXPERIMENTAL

Trypsin was crystallized by the method of Northrop and Kunitz (12). Sera of various animals were obtained with the usual technique.

Two-dimensional crossing electrophoresis was carried out at room temperature and its procedure has been described already (11, 13). Horizontal plate type apparatus* as shown in Fig. 1 was used mostly, in which a square sheet of filter paper of 20×20 cm. could be set up. The apparatus consisted of two parts: the trough and the cover, and two electrode vessels. The trough was of 18×18 cm. area and about 10 mm. deep. It was provided with a broad brim, on which the square sheet of filter paper was set up. The available area of the paper for the two-dimensional electrophoresis was about 17×17 cm.

First, the lines for the solutions to be applied were drawn on the filter paper. Then it was soaked with the buffer solution and set up on the trough. For the first electrophoresis, serum alone was applied

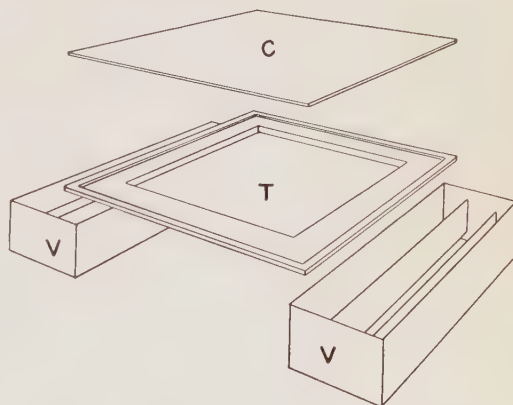


FIG. 1. Diagram of the plate type two-dimensional paper electrophoresis apparatus.

T: trough. C: cover. V: electrode vessel.
Explanation, see text.

* Obtainable from the Kayagaki Co., Tokyo.

on the predetermined line (AB). Two sheets of filter paper of 20×10 cm. were attached to the anodic and cathodic ends of the test filter paper on the trough to connect it to the buffer solutions in the electrode vessels. The trough with the filter paper was closed with the cover and the first electrophoresis was carried out in direction 1.

After the first electrophoresis the sheets of connecting filter paper were removed, the trough with the test filter paper was turned at a right angle, and the solution of trypsin was applied on the other predetermined line (XY), which was drawn perpendicularly on the anodic side (in the second electrophoresis) of the line for serum. Then the filter paper was connected with the electrode vessels, covered, and the second electrophoresis was carried out in direction 2.

Barbiturate buffer of pH 8.6 and ionic strength 0.05 and filter papers of Toyo No. 52 and Whatman No. 1 were used.

After the two runs of electrophoresis, the filter paper was dried at 100°C and stained with bromophenol blue (14) or with amidoblack 10 B (15). Optical density of the stained protein zone was measured by a densitometer.

RESULTS AND DISCUSSION

Crossing Diagram of Human Serum against Trypsin—In Fig. 2 is shown an example of "crossing diagrams" (11, 13) of human serum against trypsin. This electropherogram was obtained by the two-dimensional electrophoresis of human serum and trypsin as described above. Under the given experimental conditions trypsin migrates toward cathode and serum proteins toward anode. Thus the former migrates over the zones of the latter. As can be seen from the figure, the zones of serum proteins were partially digested by trypsin. In this way the front of the trypsin line, which is named the "crossing diagram" of serum against trypsin, appeared across the zones of serum proteins. It shows two grooves (or peaks, if viewed conversely), one in the zone of α_1 -globulin and the other in that of α_2 -globulin. At the grooves the migration of trypsin is retarded and the retardation must have been caused by a reaction with substances in serum which combined it and inhibited its activity. As trypsin migrates over the zones of serum proteins, the groove of trypsin line will become deeper where trypsin

inhibitor is present in high concentration. It is obvious that the grooves do not depend on the reaction of trypsin with any serum proteins as its substrates, because the most concentrated serum albumin did not form a

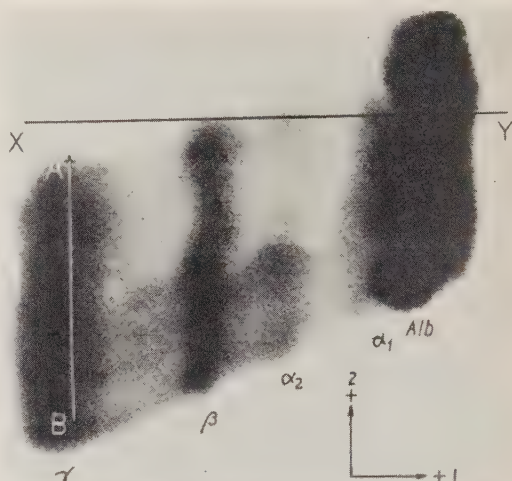


FIG. 2. Crossing diagram of trypsin inhibitors of human serum against trypsin.

First electrophoresis: 0.04 ml./6 cm. of human serum alone was applied on line AB. Electrophoresis was carried out in direction 1, at 100 volt. and 4 mA for 16 hours at room temperature. Second electrophoresis: 0.1 ml./14 cm. of 1.5% trypsin solutions was applied on line XY. Electrophoresis was then carried out in direction 2 at 100 volt. and 4 mA for 8 hours at room temperature. Barbiturate buffer of pH 8.6 and ionic strength 0.05. Filter paper, Toyo No. 52. Stained with bromophenol blue.

groove. Thus the curve obtained at the front of trypsin line would correspond to the distribution of trypsin inhibitor in the serum proteins. This conclusion may be supported also by the fact that the distribution curve obtained by Jacobsson (5) shows the same localization of two trypsin inhibitors in α_1 - and α_2 -globulins.

The line of a substance thus obtained, which corresponds to the distribution of the other substance reacting with the former has been named the "crossing diagram" of the latter* (11, 13). Therefore the curve which

* By changing the order of the two runs, two kinds of crossing diagrams can be obtained.

appeared on the front of trypsin line may be called the "crossing diagram of trypsin inhibitors" in the serum.

Distribution Curve of Trypsin Inhibitors in the Fractions of Human Serum—The crossing diagram of human serum against trypsin obtained by the two-dimensional crossing electrophoresis, however, does not necessarily coincide with the distribution curve of trypsin inhibitor. This is because the front of trypsin line does not cross each fraction of the serum at the same distance from the end, since the electrophoretic mobilities of the fractions are not the same. Nevertheless, a distribution curve of trypsin inhibitors may be drawn from its crossing diagram, provided that each inhibitor-trypsin complex had the same dissociation constant and migrated with the inhibitor.

Retardation of migration of a substance reacting with another substance in the crossing electrophoresis is analogous to that in chromatography. In chromatography a substance reacts with or is adsorbed by the stationary phase, and migrates by the addition of an eluting solvent. In the crossing electrophoresis, a substance which is applied on the filter paper perpendicularly to the direction of electrophoresis, migrates along the zone (or column) of the other substance which is applied parallel to the direction. Thus similarity of these two phenomena is obvious. Only the moving force in the crossing electrophoresis is not hydraulic but electrophoretic, and the reaction of the two substances may not only be adsorptive but sometimes chemical. Consequently the theory of chromatography (16-18) can be applied also to the crossing electrophoresis.

As discussed above, here the zones of trypsin inhibitors may be regarded as columns of chromatography for trypsin, and in the following treatment, the zone of trypsin inhibitor was supposed to be of uniform concentration. This may be realized, if a sufficiently narrow longitudinal section of the inhibitor band is considered. In formulating the equation to calculate the relative concentration of inhibitors, following symbols

were used:

$X = Vt$ = relative position of trypsin line (or band) after the migration during time, t , measured in relation to the position of trypsin inhibitor. (V is the relative electrophoretic velocity of trypsin in relation to trypsin inhibitor on the filter paper under given conditions per unit time.)

$x = vt$ = relative position of trypsin band after the migration during time, t , in the inhibitor zone. (v is the relative electrophoretic velocity of trypsin relative to the inhibitor in the zone of the latter.)

u = mobility in the first electrophoresis.

e_0 = amount of total trypsin per unit area of the trypsin zone.

e = amount of free trypsin per unit area of trypsin zone on the inhibitor zone.

i_0 = amount of total trypsin inhibitor per unit area of inhibitor zone.

i = amount of free trypsin inhibitor per unit area of inhibitor zone.

p = amount of trypsin-trypsin inhibitor complex formed per unit area on the zone of inhibitor.

K_I = dissociation constant of the complex.

Between trypsin, T , and trypsin inhibitor, I , instantaneous equilibrium

$$T + I = TI \quad \dots\dots\dots (1)$$

may be supposed to be established. Obviously

$$e_0 = e + p \quad \dots\dots\dots (2)$$

$$\text{and } i_0 = i + p \quad \dots\dots\dots (3)$$

Hence

$$K_I = \frac{(i_0 - p)e}{p} \quad \dots\dots\dots (4)$$

On the inhibitor zone, sufficiently narrow cross section of thickness, δu , can be supposed, in which $\partial e / \partial x$, $\partial e / \partial X$, and $\partial p / \partial X$ may be considered constant. When trypsin (free) migrates an infinitesimal distance, ΔX , the amount of trypsin which leaves the section across its front boundary is $(e - \Delta e)\Delta X$, and the amount which enters from its rear boundary is $e\Delta X$, provided that the trypsin inhibitor and the inhibitor-trypsin complex do not migrate in relation to trypsin. Thus the amount of trypsin carried into the section is $\Delta e\Delta X$. On

the other hand, the total trypsin in the section migrates an infinitesimal distance, Δx , on the inhibitor zone, corresponding to the distance ΔX , in the inhibitor free field. The total amount of trypsin which leaves the section across its front boundary is $(e_0 - \Delta e_0)\Delta x$ and the amount which enters from its rear boundary is $e_0\Delta x$. The difference must be equal to the amount of free trypsin carried in,

$$\Delta e_0\Delta x = \Delta e\Delta x + \Delta p\Delta x = \Delta e\Delta X \quad \dots\dots\dots(5)$$

Thus

$$\frac{\partial e}{\partial X} + \frac{\partial p}{\partial X} = \frac{\partial e}{\partial x} \quad \dots\dots\dots(6)$$

Substituting $(dX/dx)_e(\partial e/\partial X)$ for $\partial e/\partial x$, and $(dp/de)(\partial e/\partial X)$ for $\partial p/\partial X$,

$$\left(\frac{dX}{dx}\right)_e = 1 + \frac{dp}{de} \quad \dots\dots\dots(7)$$

The general solution can be written as

$$x = S(c) + \frac{X}{1 + dp/de} \quad \dots\dots\dots(8)$$

If the starting point of the experiment is chosen at the point of crossing, initial condition may be

$$X = x = 0 \quad \dots\dots\dots(9)$$

Thus

$$\frac{X}{x} = 1 + \frac{dp}{de} \quad \dots\dots\dots(10)$$

Now from equation (4)

$$\frac{dp}{de} = -\frac{i_0 K_I}{(K_I + e)^2} \quad \dots\dots\dots(12)$$

Therefore from equation (10)

$$\frac{X}{x} = 1 + \frac{i_0 K_I}{(K_I + e)^2} \quad \dots\dots\dots(13)$$

If

$$e_0 \gg i_0, \quad \dots\dots\dots(14)$$

$$e \doteq e_0, \quad \dots\dots\dots(15)$$

Hence

$$K_I/(K_I + e)^2 = A \quad \dots\dots\dots(16)$$

can be regarded as a constant, and the following equation is obtained.

$$\frac{X - x}{x} = A i_0 \quad \dots\dots\dots(17)$$

The distance, X , migrated by trypsin in relation to the inhibitor in the inhibitor free field, and that in the zone of inhibitor, x , can be measured from the crossing diagram

of trypsin inhibitor. Therefore, a distribution curve of trypsin inhibitors in the fractions of serum can be drawn.

In Fig. 3, the upper diagram shows the "crossing diagram" of trypsin inhibitors in

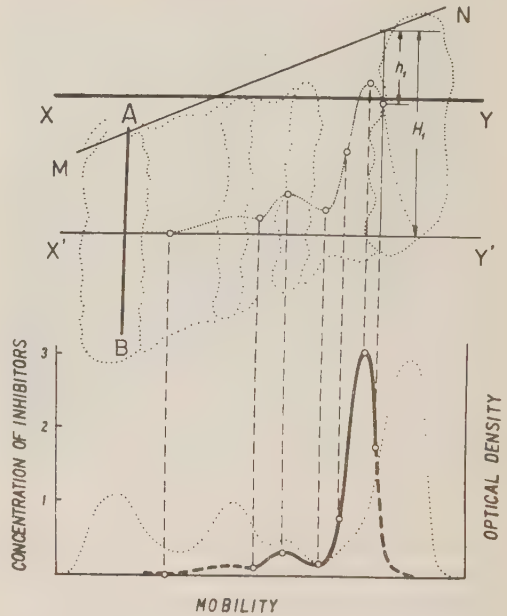


FIG. 3. Drawing of the distribution curve from the crossing diagram of trypsin inhibitors.

Upper part: Crossing diagram of trypsin inhibitor; confer Fig. 2. AB; original line of serum application. XY: original line of trypsin application. X'Y': front of trypsin line. MN: front of zones of serum proteins. Lower part: distribution curve of trypsin inhibitors in serum proteins. Fat line: distribution curve. Dotted line: electrophoretic pattern of the serum. Explanation, see text.

human serum against trypsin. Trypsin solution which was originally applied on line XY migrated up to line X'Y'. The front of the serum proteins reached line MN. Distance, H_1 , is that which trypsin migrated in relation to the corresponding fraction of serum protein. The actual distance which trypsin migrated in the fraction is denoted as h_1 . Thus the ratio

$$\frac{H_1 - h_1}{h_1} = A' i_0 \quad \dots\dots\dots(18)$$

is proportional to the quantity of trypsin

inhibitors, i_0 , in the fraction, and the lower diagram was obtained using an arbitrary value of A' as constant. It represents the curve of the concentration of trypsin inhibitors, i_0 , versus mobility, u , hence a distribution curve of trypsin inhibitors in serum proteins.

The total quantity of trypsin inhibitors, I , can be obtained by measuring the area under the distribution curve according to the equation:

$$I = A \int i_0 du \dots\dots\dots (19)$$

However, the absolute quantity of trypsin inhibitors can not be obtained by this method, and only their relative quantities can be calculated. Thus the ratio of trypsin inhibitors contained in α_1 -globulin (I_1) and in α_2 -globulin (I_2) were calculated from Fig. 3 to be about 100:16. This value falls in the range obtained by Jacobsson (5), although the quantity calculated by this method does not necessarily coincide with that obtained by other methods which measure the inhibiting activity, because the premises of each method are not the same.

By the way, it could not be decided up to present, whether a third inhibitor exists

in the region of β -globulin or not, although the presence was suspected as shown in the figure.

Crossing Diagrams of Sera of Various Animals against Trypsin—Crossing diagrams of sera of some animals against trypsin are shown in Fig. 4 to 10. A short comment which follows may be necessary.

As will be seen in Fig. 4, the crossing diagram of trypsin inhibitors of bovine serum shows only one peak in the region of α -globulin. However, since the peak is somewhat broad, it may be regarded as composed of two peaks.

The crossing diagram of trypsin inhibitors of horse serum shows two peaks (Fig. 5), one in the region of albumin and the other in the area between α - and β -globulins. It is characteristic of horse serum that its serum albumin was digested considerably by trypsin, a finding which has not been observed with sera of other animals. The distribution of the second inhibitor is somewhat broader than that of the first. The relative concentrations of the two inhibitors are nearly the same: $I_1 : I_2 = 100 : 130$.

In Fig. 6 is shown the crossing diagram of sheep serum. It shows two peaks, one in

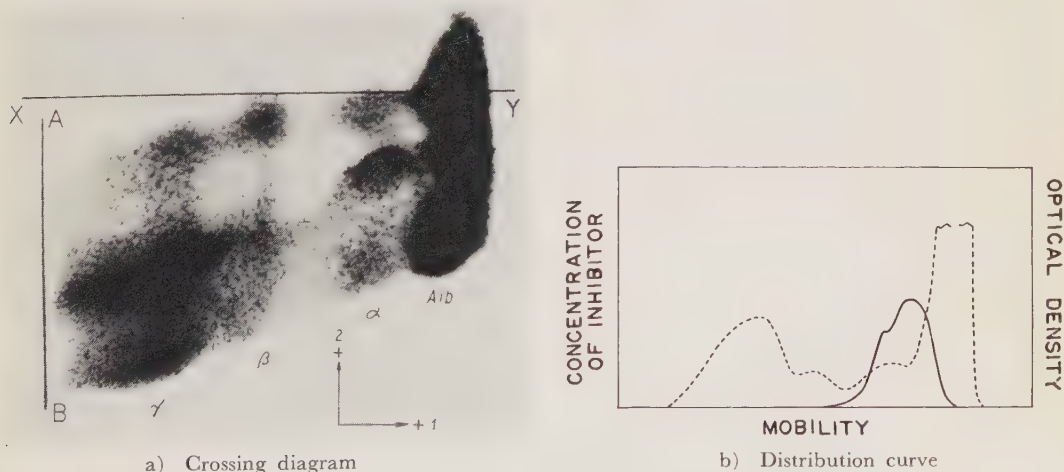
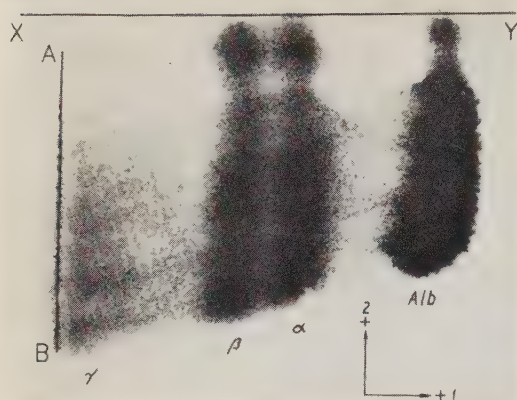
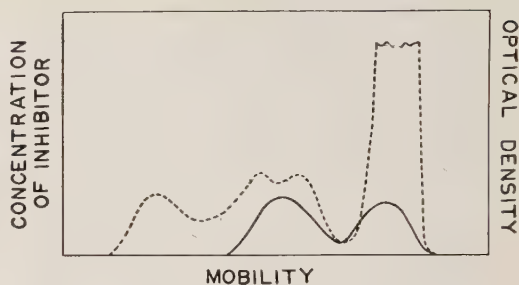


FIG. 4. Crossing diagram and distribution curve of trypsin inhibitors of bovine serum.

First electrophoresis in direction 1, at room temperature. 0.04 ml./6 cm. of bovine serum on line AB. At 100 volt. and 4 mA for 16 hours. Second electrophoresis in direction 2, at room temperature. 0.08 ml./12 cm. of 1.5% trypsin solution on line XY. At 100 volt. and 4 mA for 8 hours. Other conditions were the same as in Fig. 1.



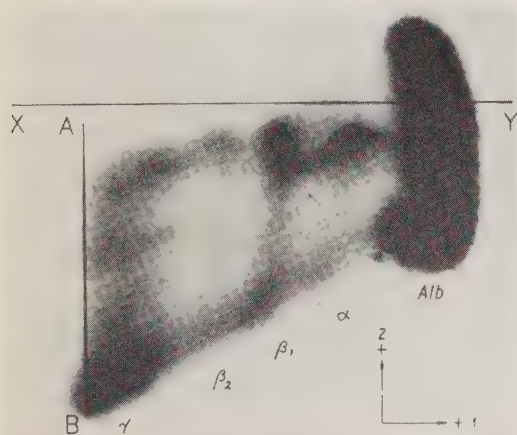
a) Crossing diagram



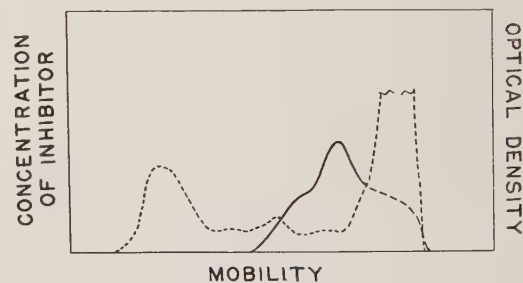
b) Distribution curve

FIG. 5. Crossing diagram and distribution curve of trypsin inhibitors of horse serum.

1st electrophoresis: 0.06 ml./8 cm. of horse serum alone on line AB. At 100 volt and 4 mA for 16 hours, at room temperature. 2nd electrophoresis: 0.1 ml./14 cm. of 1.5% trypsin solution on line XY. At 100 volt and 4 mA for 6 hours, at room temperature. Other conditions were the same as in Fig. 4.



a) Crossing diagram



b) Distribution curve

FIG. 6. Crossing diagram and distribution curve of trypsin inhibitors of sheep serum.

Experimental conditions were the same as in Fig. 5, except that the second electrophoresis was carried out for 8 hours.

the region of α - and the other in that of β -globulin. They are not completely separated. The relative concentrations of I_1 and I_2 are nearly 100 and 42, respectively. However, it seems probable that there would be one more peak in the region of albumin beside these two peaks, judged from the inclination of the crossing diagram.

The crossing diagram of trypsin inhibitors of pig serum in Fig. 7 demonstrates only one

peak in the region of α -globulin.

The crossing diagram of trypsin inhibitors of cat serum (Fig. 8) shows two peaks, one between the zone of albumin and α -globulin and the other between α - and β -globulins. The relative concentrations of the first and second inhibitors are about 100 and 41, respectively.

In Fig. 9 the crossing diagram of rabbit serum is represented. It also shows two peaks,

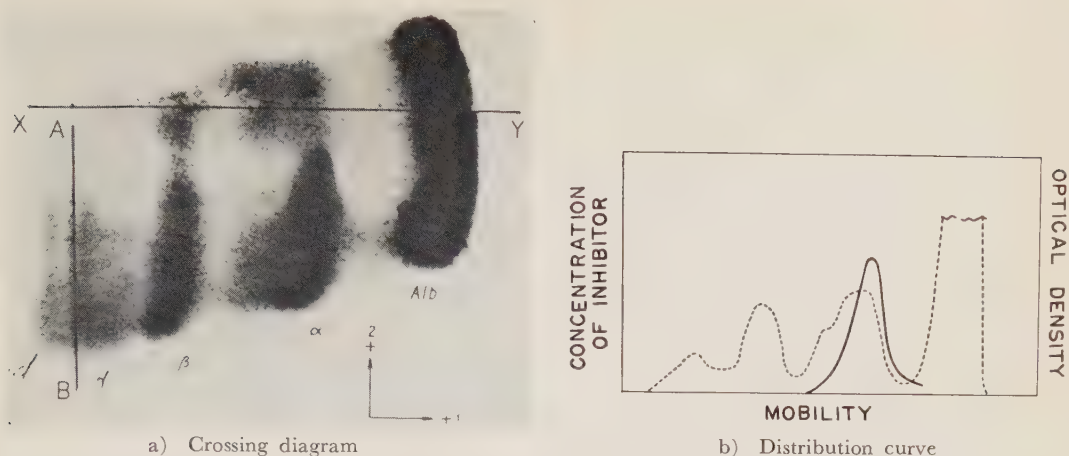


FIG. 7. Crossing diagram and distribution curve of trypsin inhibitors of pig serum. Experimental conditions were the same as in Fig. 5.

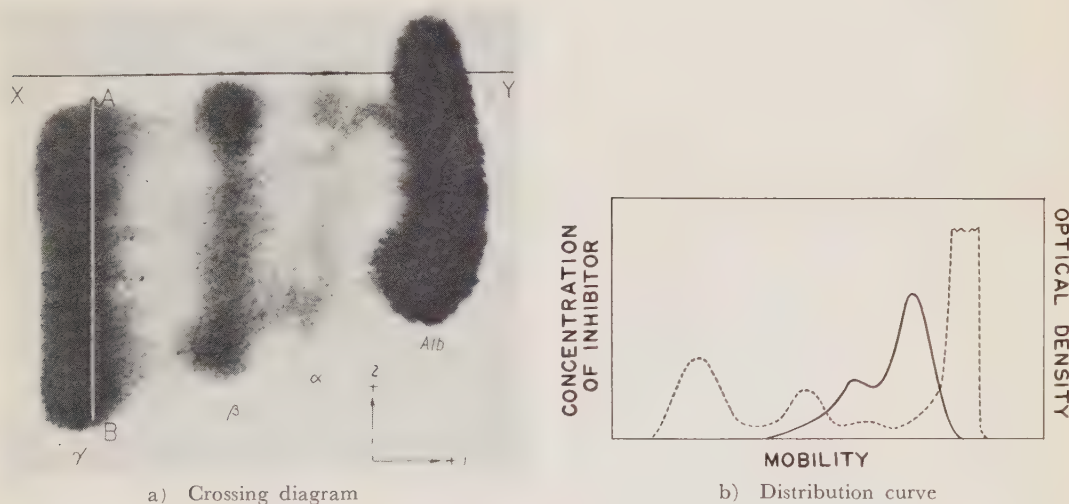


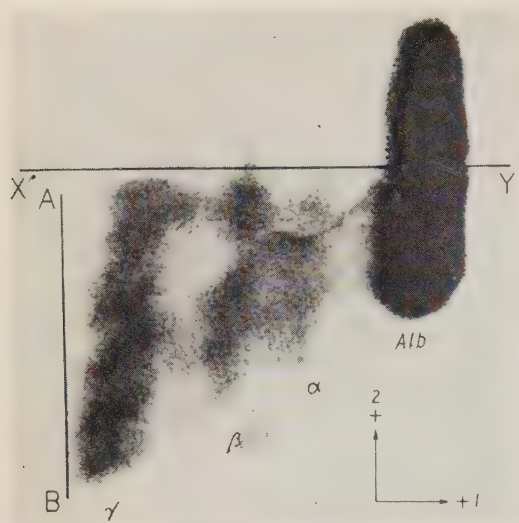
FIG. 8. Crossing diagram and distribution curve of trypsin inhibitors of cat serum. Experimental conditions were the same as in Fig. 5.

one between albumin and α -globulin and the other between α - and β -globulins. But the positions of the two peaks are slightly different from those in cat serum. The relative concentration of the first and the second inhibitors are about 100 and 60, respectively.

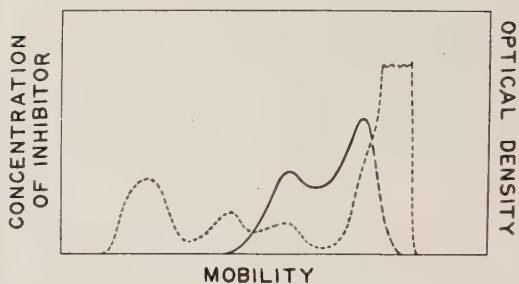
Fig. 10 shows a crossing diagram of trypsin inhibitor in guinea pig serum. Two peaks can be seen, one in the region between albumin and α -globulin and the other between α - and β -globulins. The relative concentrations of I_1 and I_2 are about 100 and 30,

respectively. Here also a third inhibitor seems to be contained in the fraction of serum albumin as in the case of sheep serum.

Previously, the distribution of trypsin inhibitors was studied only with human serum and two inhibitors, one in α_1 - and the other in α_2 -globulin, have been found by Jacobson (4, 5). They were sometimes called α_1 - and α_2 -inhibitors. Since the present study has demonstrated somewhat different distribution patterns with various animal species, it seems more logical to designate them merely



a) Crossing diagram

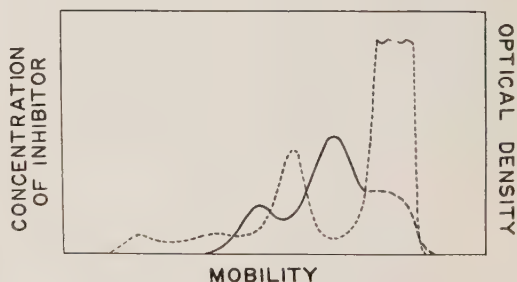


b) Distribution curve

FIG. 9. Crossing diagram and distribution curve of trypsin inhibitors of rabbit serum. Experimental conditions were the same as in Fig. 5.



a) Crossing diagram



b) Distribution curve

FIG. 10. Crossing diagram and distribution curve of trypsin inhibitors of guinea pig serum.

Experimental conditions were the same as in Fig. 5, except that the second electrophoresis was carried out for 8 hours.

I_1^- (or the first) and I_2^- (or the second) inhibitors.

Whether there is a third inhibitor in sera of some animals has not yet been conclusively elucidated, and we must await further studies.

Changes in the concentration of trypsin inhibitors in diseases were already studied by

Jacobsson (5). We have also found that the relative concentrations of the two trypsin inhibitors in human serum show individual variations, as will be reported later together with other studies on the changes in diseased conditions.

SUMMARY

Two-dimensional crossing electrophoresis of blood serum and trypsin was carried out as follows: In the first electrophoresis the serum alone was separated into its fractions. Trypsin was then applied on a line perpendicular to the serum and the second electrophoresis was carried out in the direction perpendicular to the first run. The front of trypsin crossed the zones of serum proteins and formed a characteristic curve which may show the distribution of trypsin inhibitors. Such curve has been named the "crossing diagram" of trypsin inhibitors of the serum.

Human serum showed two peaks on the crossing diagram indicating two corresponding inhibitors, one in the region of α_1 -globulin and the other in the region of α_2 -globulin.

A theory has been presented for calculating approximately the relative concentrations of trypsin inhibitors in the fractions of serum from the measurements made on the crossing diagrams of trypsin inhibitors of the serum. According to this theory, the distribution curve of trypsin inhibitors was drawn and the relative concentrations of the two inhibitors of human serum were calculated to be about 100 and 16.

Sera of cow, horse, pig, sheep, cat, rabbit, and guinea pig were also investigated by the same method, and the distribution of trypsin inhibitors in them were demonstrated. Except cow and pig, two inhibitors were observed. In the sera of sheep and guinea pig a third inhibitor was suspected.

This investigation was supported in part by a grant from the Ministry of Education.

REFERENCES

- (1) Grob, D., *J. Gen. Physiol.*, **33**, 103 (1949); **26**, 405 (1943)
- (2) Shulman, N.R., *J. Exptl. Med.*, **95**, 593 (1952)
- (3) Shulman, N.R., *J. Biol. Chem.*, **213**, 655 (1955)
- (4) Jacobsson, K., *Scand. J. Clin. Lab. Invest.*, **5**, 97 (1953)
- (5) Jacobsson, K., *Scand. J. Clin. Lab. Invest.*, **7**, Suppl., **14**, 55 (1955)
- (6) Peanasky, R.J., and Laskowski, M., *J. Biol. Chem.*, **204**, 153 (1953)
- (7) Sale, E.E., Priest, S.G., and Jensen, H., *J. Biol. Chem.*, **227**, 83 (1957)
- (8) Moll, F.C., Sunden, S.F., and Brown, J.R., *J. Biol. Chem.*, **233**, 121 (1958)
- (9) Bundy, H.F., and Mehl, J.W., *J. Biol. Chem.*, **234**, 1124 (1959)
- (10) Wu, F.C., and Laskowski, M., *J. Biol. Chem.*, **235**, 1680 (1960)
- (11) Nakamura, S., Takeo, K., and Ueta, T., *Z. physiol. Chem.*, **318**, 115 (1960)
- (12) Northrop, J.H., and M. Kunitz, M., *J. Gen. Physiol.*, **16**, 267 (1932); Northrop, J.H., Kunitz, M., and Herriott, R.M., "Crystalline Enzymes," 2nd Ed., Columbia Univ. Press, New York (1948)
- (13) Nakamura, S., Takeo, K., Katsuno, A., and Tominaga, S., *Clin. Chim. Acta*, **4**, 893 (1959)
- (14) Durrum, E.L., in "A Manual of Paper Chromatography and Paper Electrophoresis," ed. by Block, R.J., Durrum, E.L., and Zweig, G., Academic Press, Inc., New York (1955)
- (15) Grassmann, W., and Hannig, K., *Z. physiol. Chem.*, **290**, 1 (1952)
- (16) Wilson, J.N., *J. Am. Chem. Soc.*, **62**, 1583 (1940)
- (17) DeVault, D., *J. Am. Chem. Soc.*, **65**, 532 (1943)
- (18) Weiss, J., *J. Chem. Soc.*, **81**, 297 (1943)

On the Unit of Mitochondrial Structure and Function

By D. E. GREEN and T. ODA

(From the Institute for Enzyme Research, University of Wisconsin,
Madison, Wisconsin)

(Received for publication, May 18, 1961)

I. INTRODUCTION

In this communication we shall address ourselves in the main to the following questions: (1) what is the smallest common denominator of mitochondrial function and what is the size, shape, composition and organizational pattern of this hypothetical unit? (2) how can the mitochondrial unit be subdivided into smaller functional complexes, and what are the properties of these component complexes? (3) how many repeating units are present in a single mitochondrion and how are these fitted together or stacked to account for the three-dimensional structure of the mitochondrion? and (4) what is the mechanism by which electrons can flow through the component complexes of the mitochondrial unit and from one complex to the next in sequence?

During the past decade there has been a vast accumulation of information about the structure and function of the mitochondrion. However, relatively few efforts have been directed to the systematization of the available information. In our opinion the time is ripe at least to formulate the problems and to bring the issues raised above into sharper focus. Some of the data to which we shall refer has been drawn from studies carried out in our laboratory, but we are also leaning heavily on the results of other laboratories, particularly the electron microscope studies of H. Fernandez-Moran. It is our hope that the various approximate calculations which we have carried out will be a spur to further work in the field, and will encourage others to extend the scope and rigor of these first tentative efforts.

II. PRIMARY AND SECONDARY ENZYMATIC ACTIVITIES OF THE MITOCHONDRION

The mitochondrion is primarily a device for coupling energetically the aerobic oxidation of citric cycle substrates (or other substrates) to the synthesis of ATP (1). All the processes which are concerned directly or indirectly in the coupling phenomenon may be considered to be the primary enzymatic processes whereas all the rest would fall into the category of secondary enzymatic processes. The primary processes include: (1) the citric acid cycle (and related cycles); (2) fatty acid oxidation; (3) electron transport; and (4) oxidative phosphorylation (2). The secondary processes are in the main of a synthetic nature, *e.g.*, synthesis of hippuric acid (3), fatty acids (4) and phospholipids (5). In mitochondria such as those of heart, the quantitative importance of secondary enzymatic processes is relatively minor whereas in liver mitochondria the contribution is relatively much greater. The locale of the enzymes concerned in the secondary enzymatic processes has yet to be decided. Undoubtedly some of the enzymes are closely associated with the primary, particulate mitochondrial unit while others may be present in diffusible form in the spaces between cristae.

III. THE PRIMARY MITOCHONDRIAL SYSTEM

The basic functional unit of the mitochondrion is a composite of two segments: (1) a particulate, structured segment known as the electron transport particle (ETP) (6); and (2) a group of dehydrogenating complexes

which are attached to ETP but which are in large measure detachable from ETP by various mechanical (sonic irradiation) or chemical means (exposure of mitochondria to dilute ethanol or strong phosphate solution). The strength of the attachment of the various dehydrogenating complexes to mitochondrial ETP is variable so that some are more readily detached than others. The β -hydroxybutyric dehydrogenase complex (7) shows the most tenacious association with ETP while the complex of fatty acid oxidation enzymes is more loosely attached. There are five known dehydrogenating complexes: (1) the pyruvic (8), (2) the α -ketoglutaric (9), (3) the β -hydroxybutyric (7), (4) the fatty acid (10), and (5) the malic-isocitric (11), dehydrogenating complexes. The relation of these complexes to the electron transport chain of ETP is diagrammatically represented in Fig. 1.

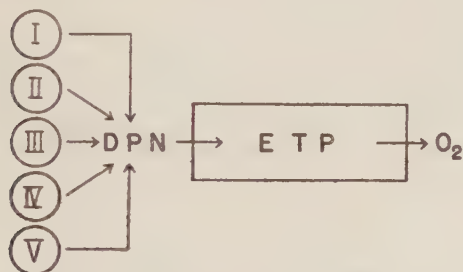


FIG. 1. A diagrammatic representation of the relation in the mitochondrial unit between the electron transport particle (ETP) and the various dehydrogenating complexes (circles with roman numerals). Bound DPN is the link between these two segments of the chain.

I	β -hydroxybutyric dehydrogenating complex		
II	pyruvic	//	//
III	α -ketoglutaric	//	//
IV	malic-isocitric	//	//
V	fatty acid	//	//

Arrows indicate direction of flow of electrons.

When these complexes are separated from ETP, electrons can no longer enter by way of citric cycle substrates or fatty acyl CoA esters but must enter by way of either succinate or DPNH. The electron flow through any of these dehydrogenating complexes is not involved in the coupling phenomena as

evidenced by the fact that ETP in its phosphorylating form shows as high a P/O ratio as does the original mitochondrion (12).

IV. THE ELECTRON TRANSPORT PARTICLE

This structured particle contains the basic oxidation-reduction protein components of the electron transport chain, *viz.* the two flavoprotein enzymes (succinic and DPNH dehydrogenases, f_s and f_D) and the four hemo-proteins (cytochromes a , b , c_1 and c) (6). In addition, coenzyme Q, non-heme iron and copper are concerned either in the interaction of these six proteins with one another or in the interaction with molecular oxygen (11). The amounts and molecular proportions of the various oxidation-reduction components in ETP are listed in Table I.

TABLE I

Concentration of Oxidation-Reduction Proteins in ETP⁽¹⁾
and Calculation of Particle Weight from
These Values

Protein	Concentration in ETP (μ moles/g.)	Molecules/unit	Calculated Particle Weight
Succinic dehydrogenase	0.32	1	3.1×10^6
DPNH dehydrogenase	0.32	1	3.1×10^6
Cytochrome a	1.30	4	3.1×10^6
Cytochrome b	0.68	2	2.9×10^6
Cytochrome c_1	0.3	1	3.3×10^6

1) Data of Linnane and Ziegler (13), Griffiths and Wharton (15) and de Bernard (14).

The molecular weight of each of the flavoproteins and cytochromes is now known with a fair degree of accuracy. Thus if we assume that the smallest common denominator of ETP (*i.e.*, the smallest molecular unit) contains only one molecule of each of the two flavoprotein it is possible to calculate the minimum particle weight of the unit from the ratio:

$$\frac{n}{[\text{prosthetic group}]} \quad (1)$$

where n is the number of moles of a given prosthetic group in one mole of subunit and

[prosthetic group] is the concentration of that prosthetic group in ETP (expressed as moles per gram of ETP). The results of such calculations are shown in Table I. The average particle weight is 3×10^6 g. The agreement between the various values obtained when the formula is applied using the concentration of each of the six oxidation-reduction proteins in turn is surprisingly good, considering some of the uncertainties in the determination of the concentration of the various prosthetic groups in ETP.

The values listed in Table I are in terms of protein dry weight. Since ETP contains 30 per cent by weight of lipid (18) the true particle weight would be 10/7 times the value of 3×10^6 , *i. e.*, 4.3×10^6 g.

There are two lines of physical evidence which are pertinent to the estimate of the particle weight of ETP. R. Bock and P. Chen (19) of the Department of Biochemistry of the University of Wisconsin have examined the sedimentation characteristics of a suspension of ETP which had been exposed to sonic irradiation for 20 minutes at 9 kilocycles per second. The sedimentation diagram disclosed a narrow distribution of particle sizes with an average sedimentation coefficient of 40 S. Assuming a frictional coefficient of 1.5, Bock and Chen estimated the particle weight to be about 3×10^6 . At present all that can be said is that if f/f_0 is of the order of 1.5 and if the population of sonicated particles of ETP is largely homogeneous, then the particle weight of ETP would be in the range of several million.

The electron microscope photographs of H. Fernandez-Moran taken of both fixed and sprayed specimens of suspensions of ETP throw some light on the shape of these particles. The photographs show a rectangular repeating unit, the long dimension of which is *ca.* 450 Å and the shorter dimension *ca.* 150 Å (average values obtained from measurements of a number of separate units). The dimensions of this unit are probably intermediate between those of a flattened disc and those of an undistorted cylinder. If the observed repeating unit is a flattened version of what was originally a right circular cylinder,

it can be estimated that the dimensions of the latter would be *ca.* 450 Å in length and 100 Å in diameter, and the approximate particle weight (calculated from formula 7 given in Appendix I) would be 2.5×10^6 g. However, if the observed repeating unit did not suffer any compression during the spraying process, it would correspond to a cylinder of about 450 Å in length and 150 Å in diameter with an approximate particle weight of 6.1×10^6 g. Thus the range of particle weight ($2.5 \times 10^6 \sim 6 \times 10^6$) would overlap with the value based on composition data (4×10^6).

It should be pointed out that the isolated rod-shaped structures which appear to be the units of ETP show a tendency to aggregate along the long dimension like sausages on a string and such aggregates may round up to form vesicles. Thus in isolated particles of ETP which have vesicular structure the units are no longer rod-shaped, but rounded. In addition, the monomeric rod-shaped particles can also form rounded or spherical particles and these particles have to be distinguished from the polymeric vesiculated particles which have structure only in the periphery.

V. DEHYDROGENATING COMPLEXES OF MITOCHONDRIAL UNIT

Three of the complexes have been isolated as well defined, high molecular weight proteins [pyruvic (21), α -ketoglutaric (22) and β -hydroxybutyric (23)] but the fatty acid and malic-isocitric dehydrogenases systems have yet to be isolated as a single entity although there are indications that these exist as such in crude mitochondrial extracts. The ultracentrifugal studies of Bock and others have shown that the pyruvic dehydrogenase complex of heart mitochondria has a M.W. of about 4×10^6 g. and the α -ketoglutaric dehydrogenase complex a M.W. of about 2×10^6 g. These complexes are probably polymers since they contain more than one molecule of prosthetic groups such as flavin dinucleotide. When the flavin-containing enzyme is detached from the complex it is found to contain only one molecule of prosthetic group per

molecule of protein (24~26). Thus the number of molecules of flavin in the pyruvic or α -ketoglutaric dehydrogenase complex specifies the exact number of monomers in the polymer. These numbers are about 10 for the pyruvic (26) and 6 for the α -ketoglutaric dehydrogenase complex (24, 26). The molecular weights of the monomeric complexes would appear to be 400,000 for the pyruvic complex (26) and 300,000 for the α -ketoglutaric complex (24, 26).

About 20 per cent of the total protein is lost in the transition from mitochondria to ETP (6). This liberated soluble protein includes in large measure the various dehydrogenating complexes but in addition, enzymes connected with secondary enzyme processes and proteins such as myoglobin. Thus the particle weight of the mitochondrial repeating unit should be 10/8 times higher than that of ETP, *i.e.*, 5×10^6 g. If we assume that the mitochondrial unit is made up of ETP associated with each of the five dehydrogenating complexes as well as with some proteins other than those of the complexes, then the experimentally determined particle weight of 5×10^6 g. would be out of line with such a mitochondrial unit. The five complexes plus additional proteins might make a contribution of at least 2.5×10^6 g. to the particle weight of ETP and the resulting unit would have a particle weight of at least 7×10^6 g. ($4.3 \times 10^6 + 2.5 \times 10^6$). This calculation suggests that the mitochondrial repeating unit is associated with perhaps no more than one dehydrogenating complex (*cf.* Fig. 2). Some such arrangement might be operative to account for the fact that fatty acid oxidation is far less extensive in heart mitochondria (27) than in liver mitochondria, and that β -hydroxybutyric dehydrogenase activity is relatively high or low depending upon the mitochondrion. Such variability can be an expression of the fact that the frequency of attachment of a given dehydrogenating complex to ETP is a variable in different mitochondria. Let us say by way of illustration that this complex is attached to 1 in 20 units in mitochondria A, 1 in 10 units in mitochondria B, and 1 in 5 units in

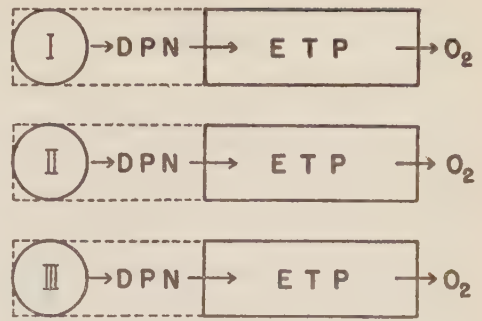


FIG. 2. Diagrammatic representation of the concept that each mitochondrial unit is associated with one or other of the various dehydrogenating complexes and not with all of them. The circles represent the dehydrogenating complexes; each complex is assigned a different roman numeral. The mitochondrial unit is a composite of ETP, bound DPN and the dehydrogenating complex.

mitochondria C. If this concept has any validity then it would mean that in a mitochondrial suspension there are qualitative differences between one mitochondrial repeating unit and another—differences which depend upon the nature of the particular dehydrogenating complex attached to a given unit. There would, therefore, be as many species of units as there are dehydrogenating complexes (assuming only one complex per unit).

VI. NUMBER OF REPEATING UNITS IN A SINGLE MITOCHONDRION

This number has been estimated by two different methods of calculation: (a) from the content of a given component of the mitochondrial unit per mg. mitochondrial protein; and (b) from the ratio of the volume of the structured elements of a mitochondrion to the volume of ETP.

For the calculation of the number of repeating units in a single mitochondrion from the content of a given component, we shall select the succinic dehydrogenase which is present in beef heart mitochondria at a concentration level of about 2.6×10^{-10} moles per mg. of mitochondrial protein. The relation between this concentration and the

number of repeating units per mitochondrion is given in the formula below.

$$\frac{[fs] \times N_A}{N_M} = N_U \quad (2)$$

where $[fs]$ is the concentration of succinic dehydrogenase in moles/mg. of mitochondrial protein, N_A is Avogadro's number (6.02×10^{23}), N_M is the number of mitochondria per mg. of mitochondrial protein and N_U is the number of units per single mitochondrion. N_M has been evaluated by the calculations described in Section VII and in Appendix II to be about 9×10^9 . Thus according to formula (2), N_U is 1.7×10^4 when $[fs]$ is 2.6×10^{-10} and N_M is 9×10^9 .

The second method for estimation of N_U depends upon the relation shown in formula (3) below:

$$\frac{V_{ST}}{V_{ETP}} = N_U \quad (3)$$

where V_{ST} is the volume of the structured elements in a single mitochondrion (including the volume occupied by the cristae and the outer or limiting membrane) and V_{ETP} is the volume of a unit of ETP (particle weight of 4.3×10^6 g.). V_{ST} has been estimated to be 1.1×10^{-13} cm.³ by the calculations described in Appendix III while V_{ETP} has been evaluated by the following formula:

$$\frac{M.W.}{N_A \times \rho} = V_{ETP} \quad (4)$$

where M.W. is the particle weight in gram of one mole of the repeating unit of ETP, N_A is Avogadro's number and ρ is the density of ETP. Assuming a value for ρ of 1.2 and a value of 4.3×10^6 for M.W. (see Appendix I for further details of these estimates), then according to formula (4), $V_{ETP} = 6 \times 10^{-18}$ cm.³ Setting $V_{ETP} = 6 \times 10^{-18}$ cm.³ and $V_{ST} = 1.1 \times 10^{-13}$, then N_U is 1.8×10^4 according to formula (3).

Thus the value of 17,000 for the number of units of ETP per mitochondrion estimated from the molar concentration of succinic dehydrogenase in mitochondria agrees well with the value of 18,000 estimated from the ratio of the volume of the structured elements of a mitochondrion to the volume of ETP.

VII. THE NUMBER OF MITOCHONDRIA FOR A GIVEN WEIGHT

The number of mitochondria per mg. of

mitochondrial protein has been estimated: (a) by direct count of the number of particles in a dilute suspension; and (b) from the ratio of the volume occupied by a given weight of mitochondria to the volume occupied by a single mitochondrion. Both methods have serious limitations but it was gratifying to find substantial agreement between the two methods that in 0.25 *M* sucrose there are between 8 and 9×10^9 mitochondria per mg. mitochondrial protein.

The direct counting of the number of mitochondria in a suspension of known mitochondrial protein with a phase microscope was carried out in chambers with an area of 0.0025 mm.² and a depth of 0.02 mm. The factor for translating mitochondria per chamber of 5×10^{-5} mm.³ volume to mitochondria per ml. of the original suspension before dilution was 2×10^{11} . When a beef heart mitochondrial suspension in 0.25 *M* sucrose with a protein content of 75 mg. per ml. was diluted 10^4 in 0.25 *M* sucrose the average number counted per chamber was 3.5 and, therefore, the number of mitochondria per mg. would be:

$$\frac{3.5 \times 2 \times 10^{11}}{75} = 9.3 \times 10^9 \quad (5)$$

The method of direct counting of mitochondria in dilute suspension is subject to the following errors: (1) Fragmented mitochondria may give rise to multiple particles; (2) not all particles will be counted; (3) any clumping of particles will lead to underestimation of numbers.

The alternative method for estimating the number of mitochondria per mg. protein is based on the following considerations and measurements. A suspension of mitochondria (10 ml.) in 0.25 *M* sucrose containing 880 mg. protein was centrifuged at 40,000 r.p.m. for 30 minutes. The mitochondrial pellet had a volume of 6 ml. and a protein content of 848 mg. Some of the protein in the original suspension was soluble and it is the loss of this protein in the supernatant (8 mg./ml.) which accounts for the decrease in the protein content of the mitochondrial pellet. The pellet may be looked upon as a composite

of mitochondria+water which has been trapped between the packed mitochondria. This water is the same as the supernatant fluid. If we assume that 50 per cent of the mitochondrial pellet represents the volume of mitochondria and the rest the volume of trapped water (*cf.* Appendix II for the basis of this estimate) then we may take it that the corrected protein content of the mitochondria would be $848 - 3 \times 8 = 824$ mg. and that this weight would correspond to 3 cm.^3 of mitochondria. Per mg. of mitochondria the corresponding volume would be $3.6 \times 10^{-3} \text{ cm.}^3$

The volume of a mitochondrion in $0.25 M$ sucrose may be computed as follows. In $0.25 M$ sucrose the mitochondrion undergoes distortion of shape from a right circular cylinder to a prolate spheroid (*cf.* diagram 1 below):

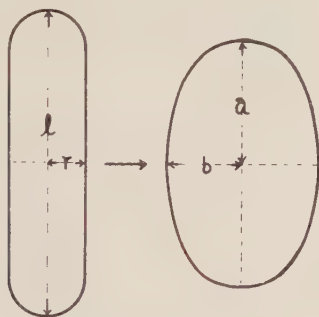


DIAGRAM 1

When sprayed or thin-sectioned specimens of fixed suspensions of beef heart mitochondria in $0.25 M$ sucrose are examined in the electron microscope the mitochondria are found to have the following dimensions— 1.3μ in the major axis and 0.8μ in the minor axis ($2a = 1.3 \mu$; $2b = 0.8 \mu$). The volume (V) of a prolate spheroid is defined by the formula shown below:

$$V = -\frac{4}{3} \pi a b^2 \quad (6)$$

Substituting the values of a and b given above (after converting μ to cm.) in formula (6), the value for V comes out to be $4.4 \times 10^{-13} \text{ cm.}^3$. When the volume occupied by 1 mg. of mitochondria ($3.6 \times 10^{-3} \text{ cm.}^3$) is divided by the volume of a single mitochondrion in $0.25 M$ sucrose ($4.4 \times 10^{-13} \text{ cm.}^3$) the quotient is the number of mitochondria in 1 mg. of mito-

chondrial protein and the numerical value of this quotient is 8×10^9 .

VIII. STRUCTURAL PROTEIN

It has long been recognized that the mitochondrion is an organized mosaic of interlocking enzymes and proteins but the key to the chemical principles which underlie this organization was missing until the isolation in our laboratory of the structural protein was announced for the first time and evidence presented that the structural protein provides the framework on which the electron transport chain is mounted (28-30).

The structural protein accounts for about 60 per cent of the total mitochondrial protein. It is isolated in the form of a colorless, water-insoluble polymer which contains no oxidation-reduction groups. At pH 10.5 or in presence of cationic detergents such as dodecyl-sulfate the structural protein (S. P.) is depolymerized to a monomeric species of M.W. 20-30,000 which can repolymerize when the pH is lowered to neutrality or when detergent is removed by dialysis. The monomeric species readily combines with the monomeric form of cytochromes a , b and c_1 to form stoichiometric complexes and also binds considerable amounts of lipid. The bonds which attach S.P. to the cytochromes and to lipid and which underlie the tendency of S.P. and the cytochromes to polymerize are probably chiefly hydrophobic bonds and it is in fact the versatility and stability of the hydrophobic bond which in large measure underlie the structural stability of the mitochondrion.

The various reagents which have been found to fragment the mitochondrion into submitochondrial particles or proteins (bile acids, lower alcohols and detergents) are reagents which are highly effective in rupturing hydrophobic bonds and this effectiveness is a consequence of the detergents competing for lipophilic areas in the molecules which are involved in the hydrophobic bond.

Studies on the properties of S.P. and of the S.P.-lipid complexes lead to a picture of the following kind for the organization of the mitochondrion. The backbone of the mito-

chondrial unit is probably a linear polymeric array of molecules of S.P. to which are attached lipid and the other protein components of the electron transport chain. As yet, it is not known how specificity of positioning and alignment is achieved but there obviously are suitable devices to accomplish this end. According to this picture, the protein molecules of the chain (with the exception of cytochrome *c*) are fixed in position and for all practical purposes must be considered to be immobile in the same sense but not to the same degree that amino acids in a protein are restricted in movement. The cytochromes and flavoproteins are in general not linked one to another but all are linked to the S.P.-lipid network. The lipid phase of the unit is probably continuous so that electron transport can proceed from substrate to oxygen in a lipid phase entirely.

Assuming that some 60 per cent of the mitochondrial protein is accounted for by S.P. (33 per cent is the actual yield by direct isolation), then in each repeating unit there would be some 100 molecules of structural protein, approximately 1,000 molecules of phospholipid, one molecule each of f_s , f_D and cytochrome c_1 , 2 molecules of cytochrome *b* and 4 molecules of cytochrome *a* (neglecting the other components of the chain). These numbers emphasize the vast disparity in numbers between the molecules of the S.P.-lipid network and the protein molecules of the electron transport chain.

IX. THE COMPONENT COMPLEXES OF ETP

The electron transport chain can be subdivided into four particles or complexes which represent the four segments of the electron

transport chain: (1) succinic-Q reductase (31); (2) DPNH-Q reductase (32); (3) QH_2 -*c* reductase (32); and (4) reduced cytochrome *c* oxidase (14, 15, 33). Each of these particles or complexes have now been isolated in highly purified form. The components of these complexes are listed in Table II and their relationships to one another and to the electron transport sequence are shown diagrammatically in Fig. 3.

Y. Hatefi and his group in our laboratory have been able to reconstruct succinic-*c* reductase, DPNH-*c* reductase and succinic (DPNH)-*c* reductase and activity as well as DPNH oxidase activity by interaction of the respective isolated particles under appropriate conditions (32, 34, 35). Thus the entire electron transport sequence from succinate and DPNH on one end to oxygen at the other can now be reconstituted from the component segments of the chain.

TABLE II.

The Components of the Four Basic Complexes of the Electron Transport System

I Succinic-Q reductase	flavoprotein (f_s) non-heme iron lipid cytochrome b^{13}
II DPNH-Q reductase	flavoprotein (f_D) non-heme iron lipid
III QH_2 -cytochrome <i>c</i> reductase	cytochrome <i>b</i> cytochrome c_1 non-heme iron lipid
IV Cytochrome oxidase	cytochrome <i>a</i> copper lipid

1) The association of cytochrome *b* with the succinic-Q reductase may be fortuitous.

From the data of Ziegler and Doeg (31), Hatefi *et al.* (32), Griffiths and Wharton (15) and Okunuki *et al.* (16).

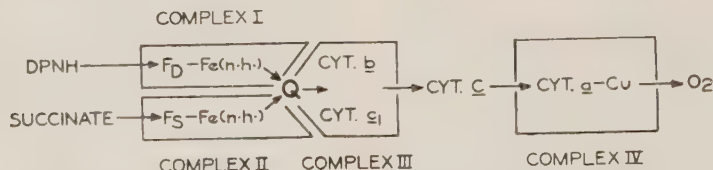


FIG. 3. The arrangement of the four component complexes of the electron transport chain. Coenzyme Q is the link between succinic-Q reductase (I) and DPNH-Q reductase (II) on the one side and QH_2 -*c* reductase (III) on the other. Cytochrome *c* is the link between III and reduced cytochrome *c* oxidase (IV).

Two of the individual complexes of the chain (succinic-Q reductase and cytochrome oxidase) have been isolated in a form the molecular weight and composition of which correspond closely with the molecular weight and composition of the pure components. For example, the M.W. of succinic-Q reductase (on a protein basis) is very nearly the same as that of the Singer-Kearney succinic dehydrogenase (36). The flavin contents are identical. The one point of difference is the presence of lipid in the former and not in the latter. Cytochrome oxidase comes close to cytochrome *a* in respect to both heme and copper content. Thus at least for succinic-Q reductase and cytochrome oxidase the amount of residual structural protein is either zero or relatively small.

However, at present both the DPNH-Q reductase and the QH_2 -*c* reductases are far from the point where the molecular weight corresponds to that of the pure component. It must be presumed, therefore, that both these particles are still associated with an excess of structural protein (perhaps 90 per cent or more by weight). The question naturally arises whether recombination of the component complexes requires the presence of S.P. in at least one of the reacting complexes. Until all the complexes are available in a form which is free of S.P. this point cannot be decided. It is conceivable that all the complexes can eventually be prepared in S.P.-free form but it is not easy to predict whether the presence of S.P. is necessary for the binding of the component complexes. This is a key question that has to be answered before the next step in the analysis can be reached. If S.P. is not required for interaction the role of S.P. is purely that of a framework protein. If S.P. is required for interaction, then the role of S.P. is a dual one—as a framework protein to which the chain is attached and as a structural component of the chain itself.

X. STACKING OF UNITS

Since there are some 15,000 repeating units per mitochondrion these obviously must

be stacked or arranged in continuous tiers like bricks in a wall. It is known that when electrons originate by way of succinate or DPNH all the cytochromes in the chain are reducible. Thus there is lateral electronic communication at least of the succinic and DPNH complexes. Particles can be prepared which show succinic-Q reductase activity and no DPNH-Q reductase activity, and vice versa. Yet as long as there is the merest trace of one or the other activity in a particle, complete reduction of the cytochromes can be effected by the addition of the substrate for the deficient activity. The only way this type of result can be explained is to assume that there is a mechanism by which electrons entering the relatively few intact chains in the particle can eventually radiate in all directions and reduce all the cytochromes in each of the chains whether or no the chain is complete. This mechanism is probably the diffusion of small molecules like coenzyme Q within the lipid medium which encompasses all chains in the particle. Any model of the stacking of mitochondrial units (*cf.* Fig. 4) must account for this phenomenon of the intercommunication of chains.

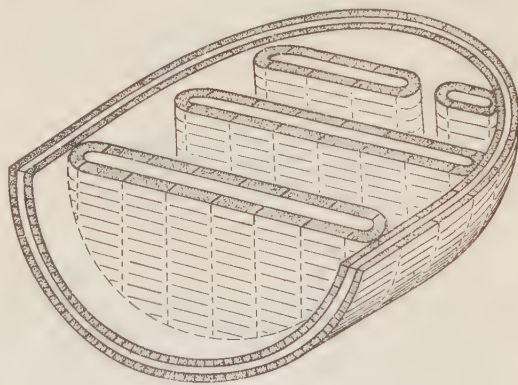


FIG. 4. Diagrammatic representation of the stacking of the repeating units within the crista and limiting membrane of a mitochondrion.

Assuming that the mitochondrial unit has the dimensions of that of ETP, *i.e.*, a cylinder of 445 Å length and 100 Å diameter, and that the additional components such as the dehydrogenating complexes do not modify

the basic geometry of the unit, the question arises whether such a unit is compatible with the structural arrangement of the mitochondrion revealed by electron microscopy. The cristae are in effect three-banded membranes of 60, 70 and 60 Å width, respectively (total width 190 Å) (37, 38) as shown in diagram 2 below:



DIAGRAM 2

Such dimensions would permit the above postulated unit to fit snugly within this membrane system as shown in diagram 3 below:

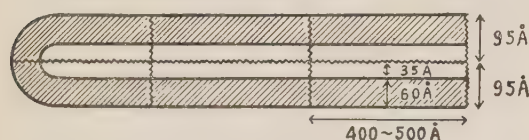


DIAGRAM 3

According to this interpretation the electron clear layer between the electron dense layers would contain elements of the unit which are of different composition than the elements in the electron dense layers. It is very probable that this layer of low electron density is the locale of lipid (*cf.* diagram 4):

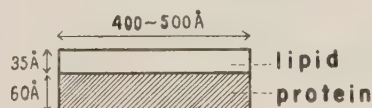


DIAGRAM 4

It will be noted that this method of stacking of units within the crista provides an internal lipid phase in which lipid soluble components like coenzyme Q have access to all units abutting on this phase.

The external membrane of the mitochon-



DIAGRAM 5

dron has a three-banded arrangement of membranes similar to that of the crista but the dimensions are somewhat smaller (37, 38) (*cf.* diagram 5):

The accommodation of the 400 Å—100 Å unit to the dimensions of the outer membranes would pose a problem because the paired units would be 50 Å wider than the overall width of the membrane. It has to be borne in mind that the isolated unit of ETP may have undergone distortion leading to an increase in the shorter dimension and a decrease in the longer dimension. The discrepancy, therefore, may not be a serious one.

If we assume that the cristae extend from the outer membrane on one side of the mitochondrion to the outer membrane on the other side (some 4,000 Å across), then a section of a crista would accommodate 4,000/400 or roughly 10 units per wall or 20 units for both walls.

XI. PAIRING OF UNITS

The mitochondrial cristae may be described as flattened tubes, the structured walls of which are usually parallel. From the way in which the two walls remain parallel in cut sections of mitochondria despite considerable distortion or displacement of the cristae it would appear that the two walls are attached to one another and that the clear region between the two walls is implicated in this attachment. We shall refer to this parallel arrangement of the membranous walls as the pairing arrangement. When mitochondria are converted to ETP the paired walls separate into particles with a single membrane character (*cf.* Fig. 5) (39). Thus the units of the mitochondrion and of ETP are probably present in each of the two paired walls or membranes. Oxidative phosphorylation is observed only in particles which retain the paired arrangement of the cristae and coupling has yet to be found in particles with unpaired membrane structure. Thus ETP_H, which is a particle with paired membrane structure, is capable of normal oxidative phosphorylation (13), whereas ETP, an analogous particle without this paired arrange-

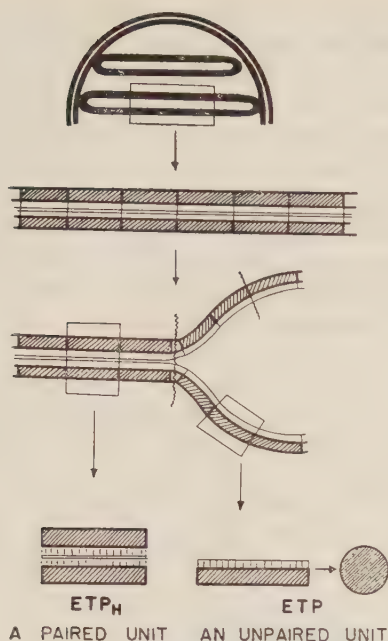


FIG. 5. Diagrammatic representation of the conversion of a crista with paired units to a membrane or particle with unpaired units. The uppermost diagram shows a portion of a mitochondrion, and the segment of one of the cristae which is outlined by the rectangle is blown up in greater detail in the diagram just below. The blocks represent individual units. The two walls of the crista can be separated and when separation is complete the paired arrangement of units is lost. ETP_H is a particle which still retains the paired arrangement of units whereas the particles of ETP do not.

ment, is lacking or deficient in this property. The precise implication of the paired arrangement has yet to be understood but it probably is related to the devices by which factors concerned in the coupling process are retained within the space enclosed by the paired membranous walls. There is no reason to suspect that the units of ETP_H are qualitatively different from those of ETP. Perhaps the addition of the appropriate factors to ETP would be sufficient to restore the capacity for oxidative phosphorylation.

XII. MECHANISM OF ELECTRON FLOW

The concept of the electron transport

chain as a composite of four complexes aligned in a definite sequence and imbedded within a structural protein-lipid network raises the question of how electrons can move within such an array. Considering the nature of the hydrophobic bond it is inconceivable that the individual components within the complex can enjoy freedom of rotation and translation. Then how do electrons move within a single complex and from one complex to another?

The problem between complexes is the easier to resolve. Coenzyme Q and cytochrome *c* are lipid-soluble molecules which are readily extractable from particles and which are clearly capable of movement within the lipid phase of particles. These could serve

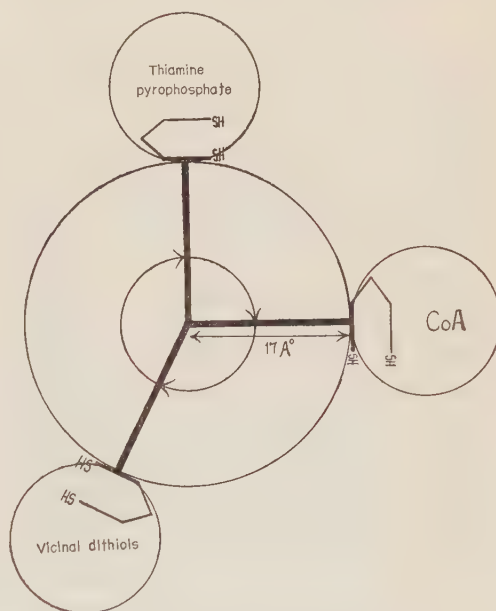


FIG. 6. Diagrammatic representation of the excursion described by the flexible arm of lipoic acid which permits the successive interaction of this coenzyme with thiamine pyrophosphate, CoA and enzyme dithiol. Each of these four prosthetic groups is localized in a different region of the α -ketoglutaric or pyruvic dehydrogenase complex. The various circles represent the area covered by the respective coenzymes—the large circle in the center of the diagram (of diameter 17 Å) being the area of revolution of lipoic acid (represented by the open 5-membered ring terminating in two sulfhydryl groups).

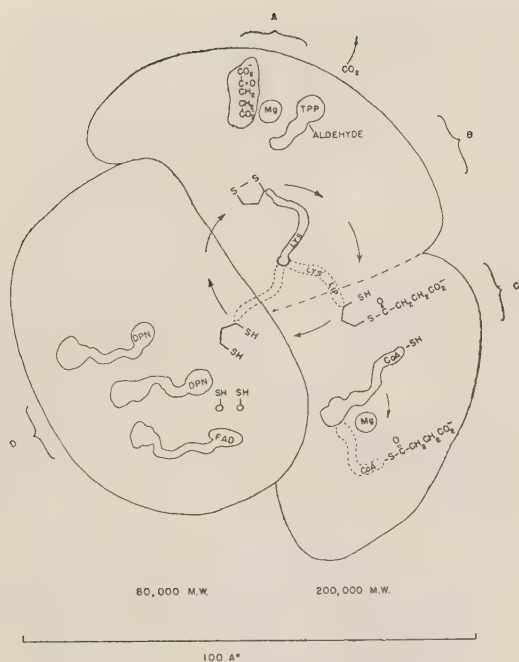


FIG. 7. A schematic representation of the central role which lysine-bound liponic acid may play in the α -ketoglutaric dehydrogenase complex drawn to molecular scale.

Region A: α -Ketoglutarate reacts with thiamine pyrophosphate (TPP) to form the succinylaldehyde adduct of TPP with release of CO₂. Region B: Oxidized liponic acid interacts with the succinylaldehyde adduct of TPP to form succinyl liponic acid and TPP. Region C: Succinyl liponic acid reacts with reduced CoA to form succinyl CoA and reduced liponic acid. Region D: Reduced liponic acid reacts with an enzyme-bound disulfide group to form oxidized liponic acid and reduced dithiol. This completes the cycle involving liponic acid. Region E: Electrons are transferred from reduced dithiol (of the liponic dehydrogenase) to FAD, and from FAD to DPN.

as mobile links in the chain—coenzyme Q between the Q reductases and QH₂-cytochrome *c* reductase, and cytochrome *c* between QH₂-cytochrome *c* reductase and reduced cytochrome *c* oxidase (40). In a similar manner DPN could serve as the mobile link between the dehydrogenating complexes and DPNH-Q reductase (*cf.* Fig. 3). The amount of translation required to effect this linking of complexes need not be extensive. All that

is needed is translation from the reducing site to the oxidizing site which could be reasonably close together.

The problem of electron flow within complexes has proved to be much more baffling. But it is possible that an hypothesis by Bock and Criddle (41) for the mechanism of electron flow through the α -ketoglutaric complex (*cf.* Fig. 6~8) may well provide a model for electron flow through fixed complexes generally.

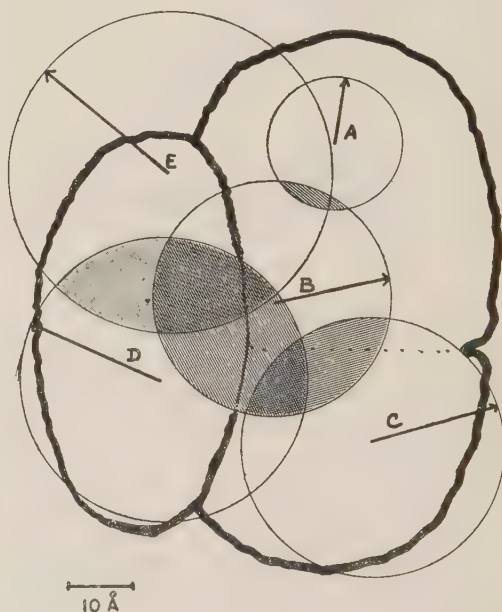


FIG. 8. Scale diagram illustrating the possibilities for collision between bound coenzymes attached to widely spaced points on the surface of the α -ketoglutaric dehydrogenase complex. The following are the keys to the various symbols:

A—thiamine pyrophosphate, B—liponic acid bound to the epsilon amino group of lysine, C—coenzyme A, D—FAD, E—DPN.

Radii of the five circles represent the distance between the functional group of the coenzyme and its point of attachment to the enzyme. It is assumed that A is bound to protein via the pyrophosphate group, B via the carboxyl of lysine and C, D and E via adenine. The protein is drawn to scale assuming a molecular weight of 80,000 for the liponic dehydrogenase and 200,000 for the rest of the α -ketoglutaric dehydrogenase. The volume assigned to protein includes water of hydration equal to 50% of the weight of protein.

The essence of the Bock-Criddle hypothesis is that electron flow within complexes is achieved by the fluctuation of oxidation-reduction prosthetic groups which are attached to the protein by flexible molecular arms such as lysine or ribosephosphate. These flexible functional groups in effect bring all the prosthetic groups within range for reaction and thus electron flow can proceed expeditiously within a complex by prosthetic group-prosthetic group interaction without it being necessary to invoke any relative movement of the component proteins.

Evidence is steadily accumulating that rapid electron flow can take place in interacting organic and inorganic systems without classical molecular collision having to be invoked. The tunneling effect in semiconductor systems (42) and the effect of bridge atoms in the interactions between inorganic metallo complexes (43) point to phenomena which may well have some applicability to the electron transport chain. Thus the abandonment of molecular collision as an explanation of electron transport in the mitochondrial system does not necessarily lead to an impasse.

APPENDIX

I. Calculation of the Particle Weight of ETP—

Proteins generally have a partial specific volume (\bar{V}) of 0.75 which corresponds to a value for the density (ρ) of 1.33 (44) since $\rho=1/\bar{V}$. The value of ρ for phospholipid is assumed to be about 0.9 at 0°C (45). Since lipid (predominantly phospholipid) accounts for 30 per cent of the dry weight of ETP, the averaged value for ρ should be equal to $(1.33 \times 0.7 + 0.9 \times 0.3)$, *i.e.*, 1.2.

The relation between particle weight (M.W.) in g. and volume (V) expressed in cm^3 and ρ as g. per cm^3 is given by formula (7):

$$\text{M.W.} = N_A \times V \times \rho \quad (7)$$

In section IV of the text it was assumed that the volume of ETP is intermediate between that of a cylinder with $l=450$ Å and $d=150$ Å and that of a cylinder with $l=450$ Å and $d=100$ Å. The volume of a right circular cylinder (V) is given by formula (8):

$$V = r^2 \pi l \quad (8)$$

If l and d are expressed in cm. the volume of the larger cylinder is $8.5 \times 10^{-18} \text{ cm}^3$ and that of the smaller cylinder is $3.5 \times 10^{-18} \text{ cm}^3$. Substituting these two values for V in formula (7), the particle weight is 6.1×10^6 g. for the larger cylinder and 2.5×10^6 g. for the smaller cylinder. Assuming a value for V intermediate between these two respective values, *i.e.*, $V = 6 \times 10^{-18} \text{ cm}^3$, the value for the particle weight of ETP comes to ca. 4×10^6 g.

II. Number of Mitochondria per Unit Weight—

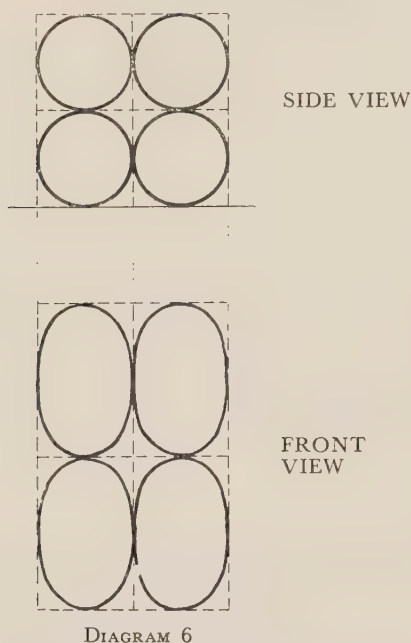
As an alternative to the method of direct counting for estimating the number of mitochondria per unit weight an effort was made to arrive at this number from the quotient:

$$\frac{V_w}{V_M} \quad (9)$$

where V_w is the volume in cm^3 occupied by 1 mg. of mitochondria and V_M is the volume in cm^3 occupied by a single mitochondrion. V_M has already been estimated to be $4.4 \times 10^{-18} \text{ cm}^3$ when mitochondria are suspended in 0.25 *M* sucrose [cf. Section VII and formula (6)]. It now remains to evaluate V_w .

When a mitochondrial suspension is centrifuged in a high centrifugal field long enough so that the volume of the sedimented pellet does not decrease any more with further centrifugation, it may be assumed that maximal packing has been achieved. In 0.25 *M* sucrose, ultracentrifugation does not lead to orientation of the particles, and, therefore, it must be presumed that in the pellet the particles are arranged randomly. When we speak of maximal packing we are not referring to the kind of packing which can be achieved with oriented particles arranged to minimize the proportion of trapped water in the interstices, but rather to the maximum achievable in a sedimented pellet in which there is a random arrangement of particles. It is not easy to compute theoretically the ratio of mitochondrial volume to the volume of trapped water in a pellet with non-oriented particles. If we assume that the mitochondrial particles although randomly arranged can, by virtue of their flexibility, approach the tight-

sucrose) according to diagram 6 shown below :



ness of packing which can be achieved by layering one particle above or below another in regular rows, then it is possible to compute the true mitochondrial volume in a sedimented pellet. Given the layering of prolate spheroids (shape of mitochondrion in 0.25 *M* then the volume of the rectangular parallelepiped which exactly envelopes the prolate spheroid is given by the equation :

$$V_p = 2a \times (2b)^2 = 8ab^2 \quad (10)$$

where V_p is the volume of the parallelepiped in cm^3 and a and b are the long and short axis of the prolate spheroid in cm . Given $a = 6.5 \times 10^{-5} \text{ cm}$. and $b = 4 \times 10^{-5} \text{ cm}$., V_p is equal to $8.3 \times 10^{-13} \text{ cm}^3$. The ratio $V_p : V_M$ multiplied by 100 represents the percent of the pellet volume occupied by mitochondria. For $V_p = 8.3 \times 10^{-13} \text{ cm}^3$ and $V_M = 4.4 \times 10^{-13}$ this percentage comes to about 50 per cent. In other words, trapped water accounts for 50 per cent of the total volume of the pellet, and packed mitochondria for the rest.

It is possible to achieve a higher percentage of trapped water (75%) by lining up particles at a 90° angle with respect to one another. However, the likelihood of such an

orientation of particles in a high centrifugal field would be very small. It is also possible to achieve a lower percentage of trapped water (*ca.* 25%) by lining up particles diagonally, instead of horizontally, but this type of orientation would be far removed from the experimental situation of a pellet with randomly oriented particles.

The estimate that 50 per cent of the total volume of the pellet obtained by prolonged high speed centrifugation of a mitochondrial suspension is accounted for by the mitochondria agrees well with what can be measured in electron microscope photographs of fixed preparations of such pellets. Planimetric measurements bear out the 1:1 volume ratio of trapped water:mitochondria.

III. *The Ratio of the Volume of Structured Elements in a Mitochondrion to the Total Mitochondrial Volume*—The mitochondrion may be considered to be a composite of structured elements and spaces between these elements (*cf.* diagram 7):

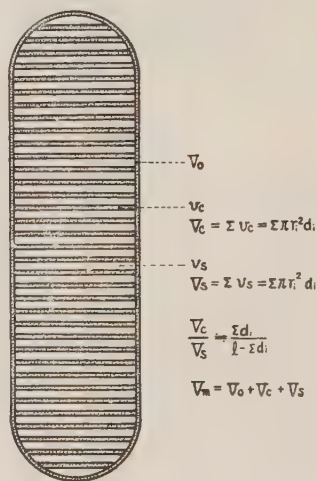


DIAGRAM 7

The formula below expresses this relation :

$$V_M = V_0 + V_s + V_c \quad (11)$$

where V_M is the volume of the mitochondrion in cm^3 , V_0 , the volume of the outer or limiting membrane, V_s , the volume of the spaces between cristae and V_c the volume of the cristae.

V_0 may be computed by the formula :

$$V_0 = 2\pi r l d \quad (12)$$

where r is the short axis of the mitochondrion (in cm.), l , the long axis, and d is the thickness of the outer or limiting membrane. Since d is 1.5×10^{-6} cm. and $r = 2 \times 10^{-5}$ cm., the error involved in using r instead of $r-d$ would be minor compared to other possible uncertainties. Using the above values for d and r and a value of 1.6×10^{-4} cm. for l , V_O comes to a value of 3.2×10^{-14} cm.³

V_M has already been computed for mitochondria in 0.25 *M* sucrose. But for our purpose it is necessary to know the value of V_M for mitochondria *in situ*, i.e., non-swollen mitochondria with the dimensions $l = 1.6 \times 10^{-4}$ cm. and $r = 2 \times 10^{-5}$ cm. Assuming that beef heart mitochondria *in situ* conforms in shape to a right circular cylinder, V_M can be computed by formulas (13) and (16) in Appendix IV and this comes to 1.8×10^{-13} cm.³

Now it remains to determine the ratio of $V_S : V_C$. By electron microscopic examination of fixed sections of mitochondria in heart muscle it is possible to determine directly the ratio of V_S to V_C . For the purposes of this type of measurement the internal cristae should be well preserved; they should be of uniform thickness and arranged in closely parallel formation preferably at right angles to the outer or limiting membrane. Measurement has shown that in a mitochondrion of 1.6×10^{-4} cm. in length there are 40 cristae from one end to the other (a proportionately smaller number in a shorter mitochondrion). The overall thickness of the individual cristae is taken to be 190 Å (37, 38). Thus in the long axis of the beef heart mitochondrion (1.6×10^{-4} cm.) there are 40 cristae, each of 1.9×10^{-6} in width (cf. diagram 7). The contribution of the outer or limiting membrane to the overall length ($2 \times 1.5 \times 10^{-6}$ cm. compared to 1.6×10^{-4} cm.) is small enough to be neglected. The ratio:

$$\frac{40 \times 1.9 \times 10^{-6}}{1.6 \times 10^{-4}}$$

is a measure of the proportion of the total interior volume occupied by the structured elements (cristae) and this value comes to about 0.5. Since $[V_C + V_S]$ is equal to 1.5×10^{-13} cm.³, V_C and V_S are equal to 7.5×10^{-14}

cm.³ and the sum of V_C and V_S is 1.1×10^{-13} .

IV. *Volume of Mitochondrion in situ*—The mitochondrion in heart muscle tissue conforms to the shape of a right circular cylinder with rounded ends at both extremities. The volume of the mitochondrion may be represented (see diagram 8) as the sum of the volume of the cylinder ($V_{cyl.}$) plus the volume of a sphere ($V_{sp.}$) (made up of two halves,

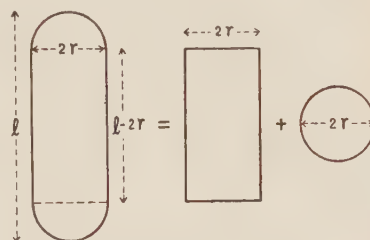


DIAGRAM 8

each from one of the extremities):

$$V_M = V_{cyl.} + V_{sp.} \quad (13)$$

If $l = 1.6 \times 10^{-4}$ cm. and $r = 2 \times 10^{-5}$ cm. and substituting in formula (14)

$$V_M = r^2 \pi (l - 2r) + \frac{4}{3} \pi r^3 \quad (14)$$

it follows that V_M equals 1.8×10^{-13} cm.³

SUMMARY

1. The following numbers have been calculated:

- the particle weight of ETP— 4×10^6 g. from composition data, $3-6 \times 10^6$ g. from electron micrograph measurements and computations, and *ca.* 3×10^6 g. from measurement of sedimentation coefficient assuming a value of 1.5 for the frictional coefficient;
- the volume of the heart mitochondria *in situ*— $0.2 \mu^3$ and in 0.25 *M* sucrose— $0.4 \mu^3$;
- the number of mitochondria per mg. mitochondrial protein— 9×10^9 by direct count and by computation of the volume of a single mitochondrion in 0.25 *M* sucrose and of the volume contribution of mitochondria in a tightly packed pellet;
- the number of repeating units in a single mitochondrion—17,000 from composition data, and 18,000 from electron micrograph data;
- the number of units in a transverse sec-

tion of a crista (4,000 Å across)—10 in each wall; the number in the entire crista—about 300;

- (f) the dimensions of ETP—a right circular cylinder in the range between 450×150 Å and 400×100 Å with a volume of 6×10^{-18} cm.³ (determined by calculation from the relation between particle weight and volume).

2. A unit of the dimensions of ETP can readily be accommodated into the membrane structure of the mitochondrion.

3. Some proposals for the mechanism of electron transport within and between the complexes of the electron transport chain have been outlined.

We are grateful to Drs. Ris and Criddle for their helpful suggestions. These investigations were supported in part by Research Grant RG-5873, Division of Research Grants (USPH; Research Grant H-458, National Heart Institute (USPH) and AEC Contract AT (11-1)-64, Project 4.

REFERENCES

- (1) Green, D. E., and Hatefi, Y., *Science*, **133**, 13 (1961)
- (2) Green, D. E., *Biol. Revs. (Cambridge Phil. Soc.)*, **26**, 410 (1951)
- (3) Sarkar, N. K., Beinert, H., Fuld, M., and Green, D. E., *Arch. Biochem.*, **37**, 140 (1952)
- (4) Wakil, S. J., McLain, L. W., Jr., and Warshaw, J. B., *J. Biol. Chem.*, **235**, 31 (1960)
- (5) Kennedy, E. P., and Bergoff, H. M., *J. Biol. Chem.*, **201**, 393 (1953)
- (6) Crane, F. L., Glenn, J. L., and Green, D. E., *Biochim. et Biophys. Acta*, **22**, 475 (1956)
- (7) Jurtshuk, P., Jr., Sekuzu, I., and Green, D. E., *Biochem. Biophys. Res. Comm.*, in press
- (8) Jagannathan, V., and Schweet, R. S., *J. Biol. Chem.*, **196**, 551 (1952)
- (9) Sanadi, D. R., Littlefield, J. W., and Bock, R. M., *J. Biol. Chem.*, **197**, 851 (1952)
- (10) Green, D. E., *Symposium Soc. Exptl. Biol.*, **10**, 30 (1957)
- (11) Green, D. E., unpublished observations
- (12) Ziegler, D., Krueger, H., and Löw, H., *Biochem. Biophys. Res. Comm.*, in press
- (13) Linnane, A. W., and Ziegler, D. M., *Biochim. et Biophys. Acta*, **29**, 630 (1958)
- (14) de Bernard, B., *Biochim. et Biophys. Acta*, **23**, 510 (1957)
- (15) Griffiths, D. E., and Wharton, D. C., *J. Biol. Chem.*, in press
- (16) Okunuki, K., Sekuzu, I., Yonetani, T., and Takemori, S., *J. Biochem. (Tokyo)*, **45**, 847 (1958)
- (17) Criddle, R. S., unpublished observations
- (18) Fleischer, S., and Klouwen, H., unpublished studies
- (19) Bock, R. M., and Chen, P., unpublished observations
- (20) Fernandez-Moran, H., unpublished studies
- (21) Schweet, R. S., Katchman, B., Bock, R. M., and Jagannathan, V., *J. Biol. Chem.*, **196**, 563 (1952)
- (22) Koike, M., Reed, L. J., and Carroll, W. R., *J. Biol. Chem.*, **235**, 1924 (1960)
- (23) Jurtshuk, P., Jr., Sekuzu, I., and Green, D. E., unpublished observations
- (24) Massey, V., *Biochim. et Biophys. Acta*, **32**, 286 (1959)
- (25) Sanadi, D. R., Langley, M., and White, F., *J. Biol. Chem.*, **234**, 183 (1959)
- (26) Reed, L. J., 'Abstracts of Papers, 139th Meeting of Am. Chem. Soc.,' March (1961)
- (27) Paul, M. H., Fuld, M., and Sperling, E., *Proc. Exptl. Biol. Med.*, **79**, 349 (1952)
- (28) Green, D. E., Tisdale, H., Criddle, R. S., Chen, P., and Bock, R. M., *Biochem. Biophys. Res. Comm.*, in press
- (29) Criddle, R. S., Bock, R. M., Green, D. E., and Tisdale, H., *Biochem. Biophys. Res. Comm.*, in press
- (30) Green, D. E., Tisdale, H., Criddle, R. S., and Bock, R. M., *Biochem. Biophys. Res. Comm.*, in press
- (31) Ziegler, D. M., and Doeg, K. A., *Biochem. Biophys. Res. Comm.*, **1**, 344 (1959)
- (32) Hatefi, Y., Haavik, A. G., and Griffiths, D. E., *Biochem. Biophys. Res. Comm.*, in press
- (33) Hatefi, Y., Haavik, A. G., and Griffiths, D. E., *Biochem. Biophys. Res. Comm.*, in press
- (34) Hatefi, Y., *Biochim. et Biophys. Acta*, **30**, 648 (1958)
- (35) Fowler, L., Haavik, A., and Hatefi, Y., unpublished observations
- (36) Singer, T. P., Kearney, E. B., and Massey, V., "Enzymes: Units of Biological Structure and Function," O. H. Gaebler, Ed., Academic Press, Inc., New York, p. 417 (1956)
- (37) Palade, G. E., "Enzymes: Units of Biological Structure and Function," O. H. Gaebler, Ed., Academic Press, Inc., New York, p. 195 (1956)
- (38) Sjostrand, F. S., *Revs. Modern. Phys.*, **31**, 301 (1959)
- (39) Ziegler, D. M., Linnane, A. W., Green, D. E., Dass, M. S., and Ris, H., *Biochim. et Biophys. Acta*, **28**, 524 (1958)
- (40) Green, D. E., *Harvey Lectures*, Ser. 52 (1956-1957)

- p. 177 (1958)
- (41) Bock, R. M., and Criddle, R. S., unpublished observations
- (42) Esaki, L., *Physicol Review*, **109**, 603 (1959)
- (43) Taube, H., and Rich, R. L., *J. Am. Chem. Soc.*, **75**, 4118 (1953)
- (44) Haurowitz, F., "Chemistry and Biology of Proteins," Academic Press, Inc., New York, (1950)
- (45) Keffler, L., and McLean, J. H., *J. Soc. Chem. Ind.*, **54**, 182 (1935)

Zur Biosynthese der Terpene XIV

Zur Bestimmung von Phosphomevalonsäure-Kinase und
Pyrophosphomevalonsäure-Decarboxylase in Zellextrakten.

Von

M. TADA und F. LYNEN

(Aus dem Max-Planck-Institut für Zellchemie, München, und dem Chemischen
Laboratorium der Universität München, Institut für Biochemie)

(Der Schriftleitung zugegangen am 19. Mai 1961)

In den letzten Jahren konnte die Biosynthese des Squalens, das als Vorstufe des Cholesterins und seiner Umwandlungsprodukte im biochemischen Geschehen der

der Mevalonsäure im Laboratorium von K. Folkers (1), die Identifizierung des seit langem gesuchten "aktiven Isoprens" mit Isopentenylpyrophosphat, die gleichzeitig im

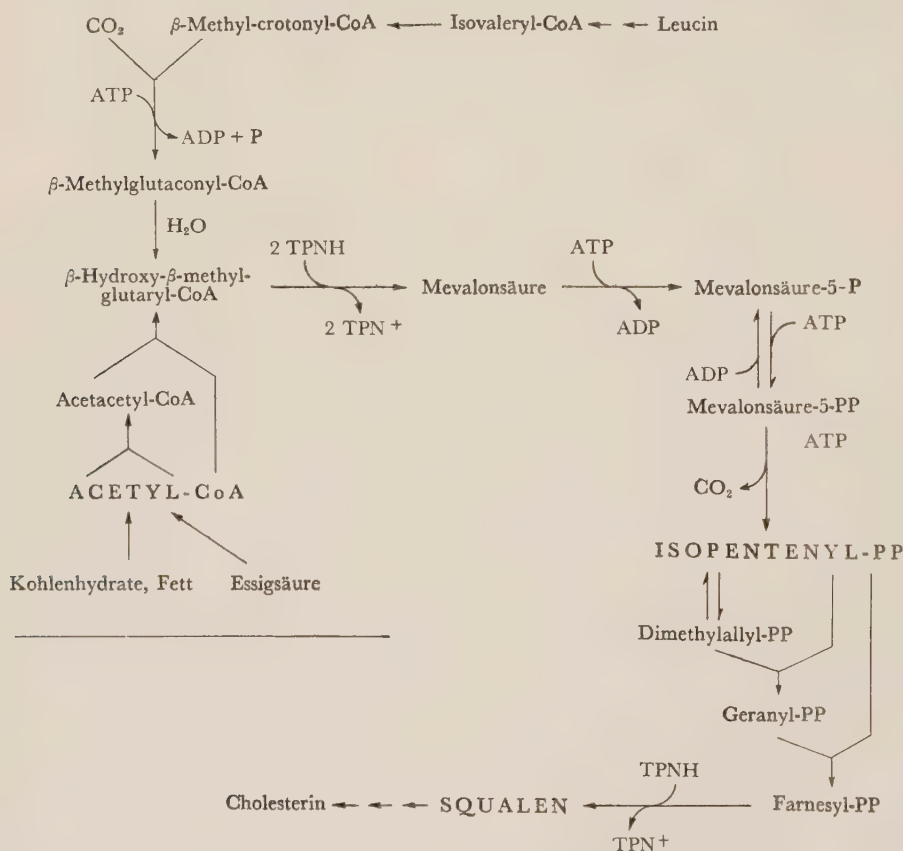


Abb. 1. Die Biosynthese des Squalens aus Acetyl-CoA oder Leucin

Säugetiere und des Menschen eine wichtige Rolle spielt, aufgeklärt werden. Meilensteine in dieser Entwicklung waren die Entdeckung

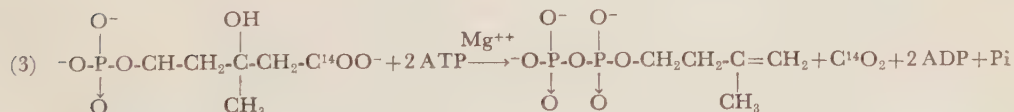
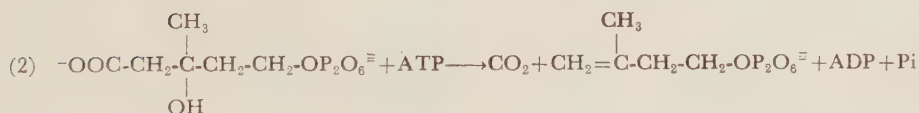
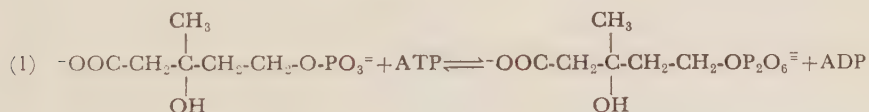
Arbeitskreis von Bloch (2, 3) und in unserem Arbeitskreis (4) gelang, und die damit zusammenhängende Entdeckung der

Allylpyrophosphate als alkylierende Agentien (4, 5). Das Resultat dieser Untersuchungen ist eine lückenlose Reaktionsfolge, durch welche sich die Umwandlung der Essigsäure über Acetyl-CoA oder des Leucins über β -Methylcrotonyl-CoA in Squalen beschreiben lässt (5). Sie ist in Abb. 1 schematisch wiedergegeben.

Mit der Aufklärung dieser Reaktionsfolge bietet sich nun die Möglichkeit, die Frage nach der biologischen Regulation der Squalensynthese experimentell zu bearbeiten, was wegen der möglichen Beziehungen zwischen Cholesterinsynthese und Arteriosklerose auch von der medizinischen Seite aus, Interesse verdient. Wir haben uns deshalb um die Ausarbeitung von Methoden bemüht, mit denen die einzelnen Enzyme dieser Reaktionsfolge nachgewiesen und quantitativ bestimmt werden können. Dabei kam es uns darauf an, Methoden zu finden, die auch auf rohe Zell- oder Organextrakte anwendbar sind und auch dort zuverlässige Werte liefern. Wir beschreiben im folgenden eine einfache Methode zur Bestimmung von Phosphomevalonsäure-Kinase (6-8) und Pyrophosphomevalonsäure-Decarboxylase (6, 7), jener

Transphosphorylierung zwischen ATP und 5-Phosphomevalonsäure, gemäss Gleichung 1, das zweite die an die Spaltung des ATP zu ADP und Orthophosphat geknüpfte Dehydratisierung und Decarboxylierung der 5-Pyrophosphomevalonsäure zu Δ^3 -Isopentenylpyrophosphat nach Gleichung 2. Den detaillierten chemischen Mechanismus dieser interessanten Reaktion konnten die Untersuchungen des Bloch'schen Arbeitskreises aufklären (6, 9). Bei den bisher beschriebenen Methoden zur quantitativen Bestimmung dieser Enzyme wurde jeweils die ADP-Bildung zur Grundlage eines optischen Tests gemacht (6-8). Man koppelte die zu messende Reaktion in bekannter Weise mit den Reaktionen der Pyruvatkinase und Lacticodehydrogenase und verfolgte den der ADP-Bildung entsprechenden DPNH-Verbrauch im Spektralphotometer bei 340 bzw. 366 $m\mu$.

Ein Nachteil dieser eleganten und nur geringen Zeitaufwand erfordernden optischen Methode, ist ihre Anfälligkeit für Störungen durch Nebenreaktionen, die beim Arbeiten mit rohen Zellextrakten so stark ins Gewicht fallen, dass die zu bestimmende Reaktion fast immer vollkommen überschattet wird. Die



beiden Enzyme durch deren Tätigkeit unter Mitwirkung von ATP 5-Phosphomevalonsäure über 5-Pyrophosphomevalonsäure in Δ^3 -Isopentenylpyrophosphat umgewandelt wird. Das erste Enzym katalysiert die reversible

optische Methode ist deshalb im allgemeinen erst nach einer gewissen Vorreinigung der rohen Enzymlösung anwendbar.

Diese Schwierigkeit entfällt bei einer Methode, deren Grundlage die Messung der

Freisetzung von radioaktivem $C^{14}O_2$ aus 1- C^{14} -Mevalonsäure-5-phosphat darstellt*, die bei der Umwandlung in Δ^3 -Isopentenyl-pyrophosphat nach Gleichung 3 eintritt.

Für diesen Prozess sind beide Enzyme erforderlich, so dass sich nach Zusatz eines der beiden Enzyme in grossen Überschuss der unbekannte Gehalt eines Zellextrakts an dem anderen Enzym ermitteln lässt.

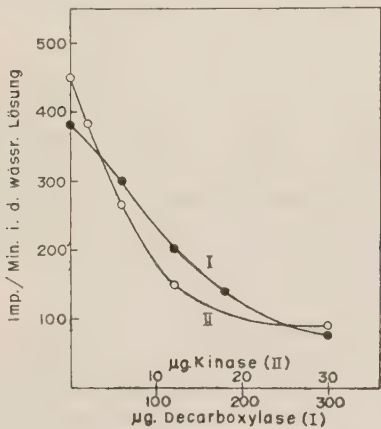


Abb. 2. Bestimmung von Phosphomevalonsäure-Kinase und Pyrophosphomevalonsäure-Decarboxylase.

Im Gesamtvolumen 0.6 ml. waren enthalten: 20 μ Mol Tris-Puffer, pH 8.0; 6 μ Mol $Mg-K_2$ -äthylendiamin-tetraacetat; 6 μ Mol $MgCl_2$; 2 μ Mol ATP; 0.1 μ Mol 1- C^{14} -Mevalonsäure-5-Phosphat (1.000 Imp./Minute); 1 mg. Serumalbumin und in I: 50 γ Phosphomevalonsäure-Kinase und die auf der Abszisse angegebenen Mengen Decarboxylase.

in II: 200 γ Pyrophosphomevalonsäure-Decarboxylase und die auf der Abszisse angegebenen Mengen Kinase.

Nach 30 Min. Inkubation bei 37°C wurde die Radioaktivität der angesäuerten wässrigen Lösung gemessen (Ordinate).

In Abb. 2 sind zwei Versuchsreihen wiedergegeben, wobei in der ersten ein Überschuss an Phosphomevalonsäure-Kinase und wechselnde Mengen Pyrophosphomevalonsäure-Decarboxylase eingesetzt wurden

* Diese Methode stammt von HENNING *et al.* (7), die sie bei Untersuchungen über die Pyrophosphomevalonsäure-Decarboxylase anwendeten.

(Kurve I), in der zweiten hingegen ein Überschuss an Decarboxylase und wechselnde Mengen Phosphomevalonsäure-Kinase (Kurve II). Die Decarboxylierung des als Substrat eingesetzten 1- C^{14} -Mevalonsäurephosphats lässt sich aus der nach der Inkubation noch vorhandenen, beim Eindampfen eines aliquoten Teils der zur Entfernung des CO_2 angesäuerten Reaktionsmischung zurückbleibenden Radioaktivität berechnen.

Als 1 m Einheit Enzym wurde diejenige Menge Enzym definiert, die unter den Bedingungen der Aktivitätsbestimmung 1 μ Mol Substrat/Minute umsetzt.

Mit Hilfe dieser Methode konnte die Anreicherung der aus Hefeautolysaten in Anlehnung an die Vorschrift von Henning *et al.* (7) gereinigten Phosphomevalonsäure-Kinase bestimmt werden. Das Ergebnis dieser Messungen ist in Tabelle I wiedergegeben.

TABELLE I
Reinigung der Phosphomevalonsäure-Kinase aus Hefe

	Protein (in mg.)	Spezifische Aktivität (mEinheiten/ mg. Protein) ¹⁾	Aktivitäts- ausbeute (in %)
Hefeautolysat (aus 200g. Hefe)	7200	3.3	(100)
Acetonfällung	3600	5	66
Nach Ansäuern auf pH 5.5	640	20	53
Elate des Al(OH) ₃ -Gels	(1.) 63 (2.) 30	67 100	30

1) 1 m Einheit Enzym setzt unter den Bedingungen der Eezybestimmung 1 m μ Mol 5-Phosphomevalonsäure/Minute um.

Wie man daraus entnehmen kann, waren die gereinigten Enzympräparate 20–30 mal aktiver als der rohe Hefeextrakt.

Eine weitere Untersuchungsreihe war der Frage nach der Verteilung der beiden Enzyme in den verschiedenen Fraktionen eines Leberhomogenats gewidmet. Wie Popjak *et al.* (10) bei Untersuchungen an den in der Zentrifuge aufgeteilten Leberextrakten fanden, sind für die Squalensynthese aus Mevalonsäure lösliche Enzyme des Überstandes und

die Mikrosomen erforderlich. Die Auswertung beider Zellfraktionen ergab, dass Phosphomevalonsäure-Kinase und Pyrophosphomevalonsäure-Decarboxylase im Überstand enthalten sind (Tabelle II). Die geringen Mengen

TABELLE II

Enzymverteilung in der Leber

Substratkonzentrationen des Standardansatzes
vgl. S. (762).

eingesetzt	m μ Mol 1-C ¹⁴ -Mevalonsäure decarboxyliert
Überstand (= 1 mg. Protein)	65
" + 50 γ Kinase	65
" + 200 γ Decarboxylase	125
Mikrosomen (= 2.6 mg. Protein)	0
" + 50 γ Kinase	0
" + 200 γ Decarboxylase	10

Phosphomevalonsäure-Kinase, die in der Mikrosomenfraktion gefunden wurden, sind wahrscheinlich auf geringe Mengen Überstand zurückzuführen, die den Partikeln noch anhafteten.

TABELLE III

*Einfluß von ATP auf den Phosphomevalonsäure-
Verbrauch im Leberextrakt*

20 μ Mol Trispuffer, pH 8.0, 6 μ Mol MgCl₂, 3 μ Mol ATP, 0.1 μ Mol 5-Phospho-1-C¹⁴-Mevalonsäure mit Leberextrakt 0.05 ml. (= 1.1 mg. Protein) im Gesamtvolumen 0.6 ml.; 3 Stunden lang bei 37°C inkubiert.

	m μ Mol Phosphomevalonsäure verbraucht
Kompletter Ansatz	88
ohne ATP	2
ohne Mg ⁺⁺	40 ^{a)}

a) Es ist zu berücksichtigen, dass bei der Bereitung des Leberextrakts eine Mg⁺⁺-haltige Pufferlösung verwendet wurde.

Die Genauigkeit der Enzymbestimmung liesse sich durch eine Anordnung, bei welcher das aus dem radioaktiven Substrat freigesetzte C¹⁴O₂ aufgefangen und gemessen wird,

wahrscheinlich noch erhöhen. Wir haben uns jedoch in der vorliegenden Untersuchung auf Grund der einfacheren Versuchstechnik mit der Bestimmung der nach dem Inkubieren der Reaktionsmischung noch verbliebenen Radioaktivität begnügt.

Bei dieser Arbeitsweise ist eine eventuelle phosphatatische Spaltung des Substrats als Fehlerquelle zu berücksichtigen. Dabei würde 1-C¹⁴-Mevalonsäure entstehen, die bei der Vorbereitung der Probe zur Radioaktivitätsmessung im Gegensatz zum Phosphorsäureester teilweise verloren gehen könnte (11). Diese Fehlerquelle lässt sich eliminieren, indem man einen Kontrollversuch mitlaufen lässt, der alle Komponenten mit Ausnahme von ATP enthält. In Abwesenheit von ATP ist die Umwandlung der 5-Phosphomevalonsäure

TABELLE IV

Der Enzymgehalt verschiedener Rattengewebe

Aktivitätsbestimmungen vgl. S. (762), wobei der Radioaktivitätsunterschied zwischen Ansätzen mit und ohne ATP ausgewertet wurde. Die Zahlen der Tabelle geben die spezifischen Aktivitäten an: d. h. m μ Mol Substrat/Min./mg. Protein des Zellextrakts.

Gewebe	Phosphomevalonsäure-Kinase	Pyrophosphomevalonsäure-Decarboxylase
Gehirn	0.28	0.22
Darm	0.09	0.20
Leber	1.28	0.44
Skelettmuskel	0.07	0.14
Niere	0.03	0.02
Hoden	0.03	0.03
Milz	0.01	0.01
Herz	0.02	0.02
Lunge	0	0
Haut		
Hefe ¹⁾	3.7	4.7

1) Messungen am Hefeautolysat (Toluol).

in Δ^3 -Isopentenyl-pyrophosphat unterbunden, so dass dann nur die phosphatatische Spaltung für einen eventuellen Radioaktivitätsverlust verantwortlich sein könnte.

Wie die Versuchsreihe mit rohem Leberextrakt (Tabelle III) zeigt, verschwindet jedoch ohne ATP-Zusatz kaum radioaktive

5-phosphomevalonsäure aus der wässrigen Phase. Die phosphatatische Spaltung des Substrats fällt somit in diesem Material neben der Decarboxylierung bei ATP-Gegenwart nicht ins Gewicht. Um aber auch bei anderen Geweben diese Fehlerquelle mit Sicherheit auszuschalten, haben wir bei der Untersuchung des Enzymvorkommens in verschiedenen tierischen Geweben, der Berechnung der Enzymaktivitäten jeweils den Radioaktivitätsunterschied zwischen den Ansätzen ohne und mit ATP zugrundegelegt. Die auf diese Weise ermittelten Aktivitätswerte verschiedener Rattengewebe sind in Tabelle IV. zusammengestellt. Bemerkenswert ist der relativ hohe Enzymgehalt des Gehirns, das unter den geprüften Geweben gleich nach der Leber kommt. Die Enzymaktivität aller Organextrakte wird jedoch von denen des Hefeextrakts beträchtlich übertroffen, so dass Hefe tatsächlich ein geeignetes Ausgangsmaterial zur Darstellung der beiden Enzyme ist (6, 7).

EXPERIMENTELLER TEIL

5-Phospho-1-C¹⁴-Mevalonsäure wurde nach Henning *et al.* (7). dargestellt.

5-Phospho-mevalonsäure-Kinase aus Hefe wurde in Anlehnung an die Vorschrift von Henning *et al.* (7) gereinigt. 200 g. Bäckerhefe (Hefefabrik Oberkotzau) wurden auf 37°C erwärmt, mit 12 ml. Toluol versetzt und 45 Minuten bei dieser Temperatur gehalten. Nach Versetzen mit 10 ml. Wasser wurde das Ganze unter häufigem Umrühren für weitere 45 Minuten bei 37°C inkubiert, dann auf Raumtemperatur abgekühlt und nach Zugabe von 180 ml. Wasser für 15–16 Stunden der Autolyse überlassen, wobei während der ersten 3–4 Stunden das pH mit 5 *M* KOH auf pH 7.4 nachreguliert wurde. Nach Abschluss der Autolyse wurden unlösliche Anteile in der Zentrifuge (20 Minuten bei 5000×*g*) abgetrennt und die überstehende Lösung mit *M* Essigsäure auf pH 6.3 gebracht. Die Fällung wurde abzentrifugiert und die Lösung (Volumen 225 ml.) mit 150 ml. gekühltem (–15°C) Aceton unter mechanischem Rühren versetzt. Der das Enzym enthaltende

Niederschlag wurde abzentrifugiert und in 40 ml. 0.005 *M* K-phosphat-Puffer, pH 7.5 gelöst. Beim Ansäuern der Lösung mit *M* Essigsäure auf pH 5.5 fiel Eiweiss aus, das abzentrifugiert und verworfen wurde. Nach Abstumpfen des pH mit *M* NH₄OH auf pH 6.7 und Abzentrifugieren von erneut ausgefallenem Protein wurde die Lösung (Vol. 32 ml., 640 mg. Protein) mit 22 ml. Al(OH)₃-Gel (Trockengewicht 320 mg.) versetzt und nach 10 Minuten langem Rühren zentrifugiert. Nach Entfernung des praktisch unwirksamen Überstands wurde der Bodensatz mit 50 ml. 0.1 *M* K-phosphat-Puffer, pH 7.5, gewaschen und die Enzymaktivität durch 3-malige Behandlung mit je 10 ml. 0.5 *M* K₂HPO₄-Lösung eluiert.

Proteingehalt und Enzymaktivitäten der einzelnen Reinigungsstufen sind in Tabelle I zu finden.

Gereinigte *Pyrophospho-mevalonsäure-Decarboxylase* aus Hefe wurde nach einer bisher unveröffentlichten Methode von Henning dargestellt. 100 ml. Hefe-Autolysat (wie zur Isolierung der Kinase bereitet; 2.3 g. Protein) wurden bei 0°C mit *M* Essigsäure auf pH 6.3 gebracht und mit 43 ml. tiefgekühltem (–15°C) Aceton unter mechanischem Rühren versetzt. Der nach Abtrennen des Niederschlags in der Zentrifuge verbliebenen überstehenden Lösung (120 ml.) wurden weitere 69 ml. Aceton unter Kühlung zugesetzt. Der Niederschlag wurde bei –10°C abzentrifugiert, in 25 ml. Wasser aufgenommen und die Lösung 6–7 Stunden lang gegen dest. Wasser dialysiert. Der dialysierten Enzymlösung (26 ml.; 935 mg. Protein) wurden 93.5 mg. neutralisierte Hefenukleinsäure (25 g. Hefenukleinsäure, Merck, in 1 Liter Wasser gelöst und mit 2 *N* KOH neutralisiert) zugefügt und die Mischung unter intensivem Rühren mit *M* Essigsäure vorsichtig auf pH 4.75 (Glaselektrode) gebracht. Nach 30 Minuten wurde der Niederschlag abzentrifugiert und die klare überstehende Lösung mit *M* TRIS auf pH 6.7 abgestumpft. Die Lösung wurde im Wasserbad für 1 Minute auf 50°C erhitzt, das denaturierte Protein abzentrifugiert und der Überstand (Vol. 28 ml.; 300 mg. Protein) für die Aktivitätsbestimmung der

Kinasepräparate eingesetzt.

Als *Versuchstiere* dienten etwa 200 g. schwere, männliche Schweizer Albinoratten. Zum Extrahieren der Gewebe wurde 0.1 M K-Phosphat-Puffer (pH 7.4) mit 0.03 M Nikotinsäureamid und 0.004 M $MgCl_2$ (12) verwendet.

Zur Bereitung der *Leberfraktionen* wurde das Organ sofort nach Entnahme aus dem durch Nackenschlag getöteten Tier in eisgekühlten Puffer gegeben und durch Dekantieren vom Blut weitgehend befreit. Das mit der Schere zerkleinerte Material wurde dann mit 2.5 Volumen Puffer versetzt und unter Kühlung im Potter-Elvehjem Homogenisator (Plexiglas) kurzzeitig homogenisiert. Zur Abtrennung von den noch intakten Zellen, den Kernen und Mitochondrien wurde 25 Minuten lang bei $10,000 \times g$ zentrifugiert. Die Mikrosomen wurden dann durch einstündiges Zentrifugieren bei $100,000 \times g$ sedimentiert, sofort noch einmal in Puffer aufgeschlämmt und dann wieder abzentrifugiert (1 Std. bei $100,000 \times g$).

Zur Bereitung der Extrakte aus *Darm, Hirn, Milz und Niere* wurden die Gewebe sofort nach dem Schlachten des Tieres in eisgekühlten Puffer gebracht, mit der Schere zerkleinert und im Potter-Elvehjem Apparat homogenisiert. Für die Aktivitätsbestimmungen wurde nur der in der Ultrazentrifuge von Partikeln befreite Überstand eingesetzt. Bei der Extraktion von *Herz, Skelettmuskel, Haut, Hoden und Lunge* wurden die durch Zerschneiden das Gewebes in eisgekühltem Puffer erhaltenen Gewebestücke zunächst im Mörser unter Zusatz von Glaspulver zerkleinert und dann erst nach Potter und Elvehjem homogenisiert. Der Proteingehalt der Extrakte wurde als Biuret nach Beisenherz *et al.* (13) bestimmt.

AUSFÜHRUNG DER ENZYMBESTIMMUNGEN

20 μ Mol Trispuffer, pH 8.0, 6 μ Mol $Mg-K_2$ -äthylendiamintetraacetat, 6 μ Mol $MgCl_2$, 2 μ Mol ATP, 0.1 μ Mol 5-Phospho-1-C¹⁴-Mevalonsäure (1000 Imp./Min.) und 50 γ gereinigte Phosphomevalonsäure-Kinase oder 200 γ gereinigte Pyrophosphomevalonsäure-Decarboxy-

lase wurden nach Zusatz der zu messenden Enzymlösung mit Wasser auf ein Gesamtvolumen von 0.6 ml. gebracht und 3 Stunden* bei 37°C inkubiert. Nach Abstoppen der Reaktion mit 0.1 ml. 3 M Trichloressigsäure wurde das denaturierte Protein abzentrifugiert, die überstehende Lösung zwecks Entfernung der überschüssigen Trichloressigsäure dreimal mit je 2 ml. wassergesättigtem Äther ausgeschüttelt und ein Aliquot der wässrigen Phase zur Radioaktivitätsmessung eingesetzt. Dazu wurde die Probe im Aluminiumschälchen unter der IR-Lampe getrocknet und dann im Methan-Durchflusszähler (Frisseke und Hoepfner, Erlangen-Bruck) ausgewertet.

Aus der verbliebenen Radioaktivität, bezogen auf die in dem Versuch eingesetzten Radioaktivität, liess sich die nach Gleichung 3 umgesetzte 5-Phospho-1-C¹⁴-Mevalonsäure berechnen.

LITERATUR

- (1) K. Folkers in G. E. W. Wolstenholme und M. O'Connor: "Ciba Foundation Symposium on the Biosynthesis of Terpenes and Sterols," J., and A. Churchill, London S. 20. (1959)
- (2) Chaykin, S., Law, J., Phillips, A. H., Tchen, T. T. und Bloch, K., *Proc. Nat. Acad. Sci. USA*, **44**, 998 (1958)
- (3) Yuan, C. und Bloch, K., *J. biol. Chem.*, **234**, 2605 (1959)
- (4) Lynen, F., Eggerer, H., Henning, U. und Kessel, I., *Angew. Chem.*, **70**, 738 (1958)
- (5) Lynen, F., Agranoff, B. W., Eggerer, H., Henning, U. und Möslin, E. M., *Angew. Chem.*, **71**, 657 (1959)
- (6) Bloch, K., Chaykin, S., Philips, A. H. und de Waard, A., *J. biol. Chem.*, **234**, 2595 (1959)
- (7) Henning, U., Möslin, E. M. und Lynen, F., *Arch. Biochem. Biophys.*, **83**, 259 (1959)
- (8) Levy, H. R. und Popják, G., *Biochem. J.*, **75**, 417 (1960)
- (9) Lindberg, M., Yuan, C. und Bloch, K., *Federation Proc.*, **20**, 230 (1961)
- (10) Popják, G., Gosselin, L., Gore, I. Y. und Gould, R. G., *Biochem. J.*, **69**, 238 (1958)
- (11) Lynen, F., Eggerer, H., Henning, U., Knappe, J., Kessel, I. und Ringelmann, E., in G. E. W.

* Bei sehr aktiven Zellextrakten wurde die Inkubationszeit auf 30 Minuten verkürzt.

- Wolstenholme und M. O'Connor: "Ciba Foundation Symp. Biosynthesis of Terpenes and Sterols," J. and A. Churchill, London, S. 95 (1959)
- (12) Bucher, N. L. R. und McGarrah, K., *J. biol. Chem.*, **222**, 1 (1956)
- (13) Beisenherz, C., Boltze, H. J., Bücher, Th., Czok, R., Garbade, K. H., Meyer-Arendt, E. und Pfeleiderer, G., *Z. Naturforsch.* **8 b**, 555 (1953)

An O¹⁸ Study of the Hemoglobin Degradation to Biliverdin in the Model Reaction*

By F. K. ANAN and H. S. MASON†

(From the Department of Biochemistry, Tokyo Medical and Dental University, Tokyo and the †Department of Biochemistry, University of Oregon Medical School, Portland, Oregon, U. S. A.)

(Received for publication, February 28, 1961)

The mechanism of oxidative degradation of hemoglobin (Hb)-hem to biliverdin is known to involve a series of oxidative reaction steps. At least two intermediates are formed in the *in vitro* experiments; they have been characterized mainly by their spectroscopic properties, and are the oxy(porphyrin)-hem-Hb** and verdohem-globin** (Lemberg, 1956 (1)).

The O¹⁸ isotopic experiments were carried out to study the chemical mechanism of biliverdin formation from Hb-hem by model reaction(s) which, however, may differ from the *in vivo* or TPN-dependent enzymatic oxidation (Nakajima (2)).

Reaction mixtures consisted of 80 ml. of Hb solution (100 μ moles) at pH 7.6 (0.1 M phosphate buffer) and 20 ml. of neutralized ascorbate solution (10 moles) in 0.1 M phosphate buffer, pH 7.6. The ascorbate was added in 4 portions at 30 minute intervals from a separatory funnel, the contents of which were being gassed with hydrogen. At the end of the incubation period (2 hours) at 50°C, the mixture was frozen, lyophilized, and biliverdin isolated by the method of Lemberg *et al.* (3). For the O₂¹⁸ experiments the gas phase was repeatedly evacuated

and filled with O₂¹⁸ from a reservoir***.

The purity of labelled biliverdin isolated (3) and purified according to the Lemberg's method (4) was 90 to 95 per cent when determined spectrophotometrically at 655 m μ (the absorption maximum). The shape of the absorption curve of O¹⁸-labelled compounds were identical with that of a standard biliverdin****. Contamination of the oxidized derivatives of biliverdin (bilipurpurins and biliviols) was nil so far as the fluorescent test upon adding alcoholic Zn reagent (Kench (5)).

The O¹⁸ content of the biliverdin samples was determined by Unterzaucher analyzer followed by mass spectroscopy***** of the recovered carbon dioxide (6). Atom per cent excess of oxygen in a reservoir obtained electrolytically from H₂O¹⁸ was determined by the direct mass spectroscopic analysis*****.

The results thus indicate approximately 1 atom of net incorporation of O¹⁸ from molecular oxygen. Since the biliverdin isola-

*** O¹⁸-enriched water was electrolyzed after metallic Li being added, and molecular oxygen generated was stored in an aspirator-type bottle filled with pure liquid paraffin and water. Hydrogen evolved was collected in two aspirator bottles of the same size as oxygen reservoir. The levels of liquid paraffin overlying on water were balanced from time to time by arranging the barrel connected to each bottle through their outlet mouth.

**** Prepared by the method of Lemberg (4) from bilirubin. The fresh biliverdin was used for standardization of its absorption curve which was consistent with that in the references.

***** Determined by Dr. C. C. Delwiche at the University of California, Berkeley.

* This work was supported by a grant from the United States Public Health Service.

** Oxyporphyrin-hem is used for the C₃₄-intermediate having —OH or =O at α -methine carbon of hem. Verdohem stands for the C₃₃ compound, in which α -methine carbon is replaced by —O—, but practically the crude cholehem obtained at the incubation product in the model experiment is accompanied by biliverdin-iron globin, containing —O—H··O= between pyrrole rings I and IV.

TABLE I

Analysis of O^{18} Incorporated in Biliverdin

(A) O^{18} used	(B) O^{18} detd. as CO_2^{18}	(x) O^{18} incorpd.
atom % excess	atom % excess	atoms per mole
9.4 ₆	0.68 ₀	0.8 ₆
8.9 ₇	0.77 ₀	1.0 ₃
8.9 ₇	0.88 ₅	1.1 ₈

Average 1.0₂

O^{18} incorporation was calculated according to the following equation:

$$x = \frac{B}{A} \times 2n$$

whereby n stands for the number of oxygen atom in the compound tested.

tion procedure allows exchange of solvent oxygen with oxygen-containing group(s), particularly during acetic acid treatment, ordinary (unlabelled) lyophilate was treated with a glacial acetic acid- H_2O^{18} mixture (2:1) to measure the extent of this exchange. The O^{18} incorporation from water during acid treatment was determined as 1.2₂-1.5₉ (average 1.4₁) atoms per mole.

It is thus possible that two or more atoms of molecular oxygen were incorporated into the biliverdin precursor(s) contained in the lyophilate, and that all but one atoms of incorporated oxygen were exchanged out during acid treatment for purification.

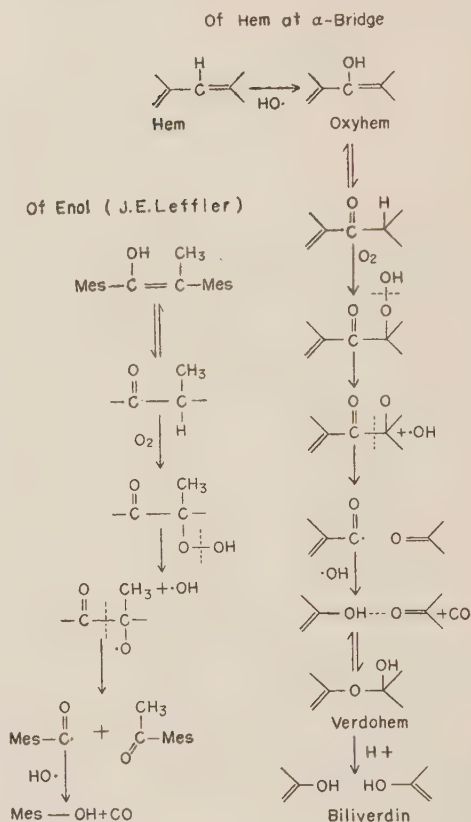
The following reaction mechanism, involving free radical reactions, is tentatively proposed in analogy with the autooxidative degradation (7) of an enol compound (8).

The indicated liberation of carbon monoxide has been reported by Sjoestrand (9), but O^{18} determination in carbon monoxide formed in the ascorbate model-reaction was not attempted because of technical difficulties. On the other hand, the autooxidative conversion of oxyporphyrin-hem (or so-called 630 $m\mu$ -substance of Kazi (10)) to verdohem or biliverdin-iron complex has been confirmed by many authors (11). Therefore, the series of reactions shown in Scheme 1 is consistent with previous observations (11).

The system is under further study by electron spin resonance spectrometry.

SCHEME 1

Proposed Mechanism of Autooxidation



REFERENCES

- (1) Lemberg, R., *Rev. Pure & Appl. Chem.*, **6**, 12 (1956)
- (2) Nakajima, H., *Proc. Japan Acad.*, **34**, 712 (1958); **35**, 144 (1959); '33th General Meeting of Japanese Biochemical Society', November (1960)
- (3) Lemberg, R., Lockwood, W. H., and Legge, J. W., *Biochem. J.*, **35**, 363 (1941)
- (4) Lemberg, R., and Legge, J. W., *Austr. J. Exptl. Biol. Med. Sci.*, **18**, 95 (1940)
- (5) Kench, J. E., Gardikas, C., and Wilkinson, J. F., *Biochem. J.*, **47**, 129 (1950)
- (6) Doering, W. E., and Dorfman, E., *J. Am. Chem. Soc.*, **75**, 5595 (1953)
- (7) Leffler, J. E., *Chem. Rev.*, **45**, 385 (1949)
- (8) Fuson, R. C., Byers, D. J., Rachlin, A. I., and

- Southwick, P.L., *J. Am. Chem. Soc.*, **64**, 2886 (1942)
- (9) Sjostrand, T., *Acta Physiol. Scand.*, **26**, 328 (1952)
- (10) Kaziro, K., and Kikuchi, G., *J. Biochem.*, **38**, 213 (1951)
- (11) Lemberg, R., and Legge, J.W., "Hematin Compounds and Bile Pigments," Interscience Publ. New York, U.S.A., p. 117 (1949)

On the Deamination of Phosphoryl-ethanolamine in Rat Liver Homogenate

As previously reported by Horie and Terada (1), and Horie (2, 3), the perchloric acid extracts from liver of rats injected intraperitoneally with P^{32} -inorganic phosphate were found to contain radioactive phosphoryl-ethanolamine and phosphoryl-choline. These extracts were also found to contain cytidine diphosphate-ethanolamine and cytidine diphosphate-choline showing far less radioactivity. These basic phosphoric esters were not adsorbed on Dowex 1 formate resin (X10, 200-400 mesh). However, the basic esters could be adsorbed on Dowex 50 (H-form, X4, 200-400 mesh) and eluted separately by the use of a hydrochloric acid gradient. The P^{32} -labelled fractions thus obtained were identified by paper chromatography.

The purified P^{32} -labelled phosphoryl ethanolamine was incubated with a rat liver homogenate at pH 7.4 in Tris buffer under aerobic conditions. The homogenate was prepared by homogenizing rat liver tissue with 9 volumes of 0.25 M sucrose solution. After the incubation perchloric acid was added. The acid soluble supernatant was found to contain very little inorganic P^{32} and most of the added P^{32} -phosphoryl ethanolamine could be recovered unchanged. Thus the ethanolamine phosphate showed a remarkable resistance to hydrolysis of the phosphate ester under the experimental conditions. However, when α -ketoglutarate was added to the reaction mixture, two radioactive fractions other than the original phosphoryl ethanolamine were found by the successive chromatography first on the Dowex 1 and then on the Dowex 50 column.

A fraction which was not adsorbed on both Dowex 1 and Dowex 50 was labelled fraction x_1 . The amount of this fraction varied considerably from experiment to ex-

periment. The other fraction (x_2) was adsorbed on the Dowex 1 column and was eluted in the same manner as a sugar phosphate or glycerophosphate (1) indicating that it contained neither basic nor carboxyl groups (Fig. 1). The amount formed was relatively

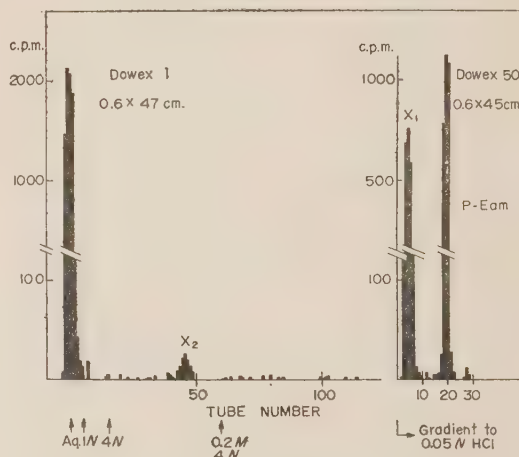


FIG. 1. Gradient elution chromatography of the reaction product.

The reaction mixture contained P^{32} -phosphoryl ethanolamine (20 μ moles) and α -ketoglutarate (50 μ moles).

Arrows indicate the positions where the solution in the reservoir was changed.

↑ Aq: distilled water

↑ 1 N: 1 N formic acid

↑ 4 N: 4 N formic acid

↑ 0.2 M, 4 N: 4 N formic acid containing 0.2 M ammonium formate

Mixer volume: 150 ml., 1 tube: 4 ml.

P-Eam: phosphoryl ethanolamine

small and seemed to depend upon the amount of added α -ketoglutarate (Table I). The fraction x_2 was collected, lyophilized, and studied with paper chromatography.

In order to further identify x_2 , its mobility was compared chromatographically with

TABLE I

Formation of Fraction x_2 from Phosphoryl Ethanolamine

No. of Experiment	P ³² -Phosphoryl Ethanolamine with Carrier	α -Keto-glutarate	Fraction x_2	% Yield of Fraction x_2
1	20 μ moles (5,300 c.p.m.)	0	0	0
2	20 μ moles (30,700 c.p.m.)	50 μ moles	448 c.p.m.	1.46
3	50 μ moles (72,450 c.p.m.)	500 μ moles	2,548 c.p.m.	3.54

Incubation: 38°C, 60 minutes, with shaking under air.

Reaction mixture:

10% rat liver sucrose homogenate	4.0 ml.
reactants dissolved in 0.05 M Tris buffer (pH 7.4)	3.8 ml.
0.2 M MgCl ₂	0.2 ml.
total fluid volume	8.0 ml.

ethylene glycol-monophosphate and glycolaldehyde-phosphate. These compounds were chemically prepared. The chemical synthesis of ethylene glycol-phosphate was carried out either by direct phosphorylation of ethylene glycol with phosphoric acid and phosphorus pentaoxide following the modified method of Plimner (4), used for the synthesis of O-phosphoryl serine, or by the deamination of phosphoryl ethanolamine with sodium nitrite. The chemical synthesis of glycolaldehyde-phosphate was carried out as follows:

P³²-phosphoryl ethanolamine with carrier (50 μ moles) was dissolved in 5 ml. of water, added with 5 ml. of the Ninhydrin-Hydrindantin Reagent (5), heated in a boiling water bath for 15 minutes. After cooling, 10 ml. of water was added and extracted twice with 20 ml. of *n*-butanol to remove the dye. The faintly colored aqueous layers were dried in a vacuum desiccator over CaCl₂ and NaOH, redissolved in 10 ml. of water, filtered, and chromatographed on the Dowex 1 formate column. The P³²-phosphoryl glycolaldehyde

fraction thus obtained was lyophilized and employed as a standard. The yield was approximately 70 to 80 per cent.

Both ethylene glycol-monophosphate and glycolaldehyde phosphate were eluted from the Dowex 1 formate column in the same position as x_2 .

On paper chromatographic examinations, x_2 and synthetic phosphoryl glycolaldehyde gave the same R_f values with three different solvent systems (Table II). It should also be

TABLE II

Identification of x_2 with Paper Chromatography

	Solvent	x_2	Phosphoryl-glycol-aldehyde	Phosphoryl-glycol
R_f	Methanol-conc. Formic Acid-Water (80 v : 15 v : 5 v)	0.76	0.75	0.80
	<i>t</i> -Butanol-Picric Acid-Water (80 ml. : 2 g : 20 ml.)	0.66	0.66	0.74
	Phenol-Water (72 v : 28 v)	0.53	0.53	0.51
	<i>m</i> -Phenylenediamine Spot Test	+	+	-

noted that both phosphoryl glycolaldehyde and x_2 gave a positive spot test with meta-phenylene diamine spray reagent. These results suggest that phosphoryl ethanolamine was either transaminated or deaminated on being incubated with the rat liver homogenate in the presence of α -ketoglutarate, and the probable reaction product was phosphoryl glycolaldehyde.

REFERENCES

- (1) Horie, S. and Terada, S., *J. Biochem.*, **47**, 335 (1960)
- (2) Horie, S., *J. Biochem.*, **47**, 429 (1960)
- (3) Horie, S., *J. Biochem.*, **49**, 116 (1961)
- (4) Plimner, R. H. A., *Biochem. J.*, **35**, 461 (1941)
- (5) Moore, S., and Stein, W. H., *J. Biol. Chem.*, **211**, 907 (1954)

Department of Biochemistry
Faculty of Medicine
University of Tokyo, Tokyo

SHIGEO HORIE
NORIO SHIMAZONO

(Received for publication, March 7, 1961)

INDEX TO AUTHORS

A

- Abe, S. See *Kikuchi, Abe and Muto* 570
 Akamatsu, S. and Watanabe, T. The Quantitative Determination of Arginine 566
 Akashi, S. and Ishihara, H. Studies on the Amino Acids Present in Yeast RNA in bound form. II. Liberation of Amino Acids from RNA by Alkaline Treatment 481
 Anan, F.K. and Mason, H.S. An O¹⁸ Study of the Hemoglobin Degradation of Biliverdin in the Model Reaction..... 765
 Ando, T. and Sawada, F. Studies on Protamines. IX. Fractionation of Clupeine 252
 Asada, T., Masaki, Y., Kitahara, K., Nagayama, R., Hatashita, T. and Yanagisawa, I. Conversion of Prothrombin into Thrombin. I. DEAE-Cellulose Chromatography of Prothrombin and Isolation of a Prothrombin Derivative. 721
 Asahi, T. See *Kawashima and Asahi* 52
 Aso, Y. See *Miura, Furiya, Aso, Imadome, Nishimura and Urushibara* 519

B

- Bosson, Gh. See *Jeuniaux, Duchâteau-Bosson et Florkin* 527
 Brubacher, G. See *Wiss und Brubacher* 609
 Buchanan, J.M. See *Takeyama and Buchanan* ... 578

C

- Chibata, I., Yamada, S., Ito, H., Furutsu, S. and Utsumi, I. The Relation of Alanine and Vitamin B₆ to Growth of Lactic Acid Bacteria. 373

D

- Duchâteau-Bosson, Gh. See *Jeuniaux, Duchâteau-Bosson et Florkin* 527

E

- Ebata, M. Action of Trypsin upon Glutamic Acid- γ -hydrazide Derivative 110
 Egami, F. See *Takahashi and Egami* 358
 —. See *Kishimoto, Takahashi and Egami*..... 436
 Eidoh, F. See *Hakomori, Ishimoda, Kawauti and Eidoh* 307

F

- Fitzpatrick, T.B. See *Seiji and Fitzpatrick* 700
 Florkin, M. See *Jeuniaux, Duchâteau-Bosson et*

- Florkin* 527
 Fu, T. See *Ota, Fu and Hirohata*..... 532
 Fujino, M. See *Kuroda, Karazumi, Fujino, Tani, Nagai and Matsuzawa* 693
 Fujino, R. See *Matsumura and Fujino*..... 561
 Fujioka, M., Morino, Y. and Sakamoto, Y. Enzymatic Studies on Pyridoxine Metabolism. V. Enzymatic Reduction of Isopyridoxal 333
 Fujita, Y. γ -Hydroxyarginine, a New Guanidino Compound from a Sea-cucumber. III. Actions of Arginase and Arginine Decarboxylase 468
 Fujita-Ikeda, S. Urea Denaturation of Takamylase A. II. Kinetic Aspects 267
 Fujita-Ikeda, S. See *Isemura and Fujita-Ikeda* ... 278
 Fujiwara, T. Studies on Chromoproteins in Japanese Nori, *Porphyra tenera*. V. On the Sugar Components of Phycoerythrin 361
 Fukutome, K. Biosynthesis of Isovalthine in the Ca⁺ 444
 Fukuyama, T. See *Sato, Fukuyama, Suzuki, Yoshikawa and Gorrod*..... 164
 Funatsu, M. See *Tokuyasu and Funatsu* 297
 Furiya, S. See *Miura, Furiya, Aso, Imadome, Nishimura and Urushibara* 519
 Furutsu, S. See *Chibata, Yamada, Ito, Furutsu and Utsumi* 373

G

- Gorrod, J.W. See *Sato, Fukuyama, Suzuki, Yoshikawa and Gorrod*..... 164
 Goto, M. See *Miura, Okamoto and Goto* 508
 Green, D.E. and Oda, T. On the Unit of Mitochondrial Structure and Function..... 742

H

- Hakomori, S. and Ishimoda, T. Presence of Formyl-residue as the Form of N-Formyl-Hexosamine in the Urinary Glycoprotein 264
 Hakomori, S., Ishimoda, T., Kawauti, H. and Eidoh, F. Isolation, Characterization of an Abnormal γ -Glycoprotein from Urine of Patients with Cancer..... 307
 Haruna, K. Fate of Some Sedatives in the Animal Body. I. α -Bromoisovalerylurea (Bromural) 388
 Hashimoto, A. See *Kajita, Hashimoto, Okazaki and Kaziro*..... 538
 Hashimoto, T., Nakao, M. and Yoshikawa, H. Some Properties of Muscle Glycerate 2,3-Di-

H (cont'd)

- phosphatase and Activation by Mercuric Ion.. 595
 Hatashita, T. See *Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa*..... 721
 Hazama, H. See *Kitagawa, Hazama, Motomura, Yamamura, Sawada and Nagai*..... 477
 Higashi, T., Yagi, M. and Hirai, H. Immunochemical Studies on Catalase. I. Assay of Catalase in Erythrocytes and Liver..... 707
 Hirai, H. See *Higashi, Yagi and Hirai* 707
 Hirai, H., Sekine, K., Iijima, A. and Warabioka. Some Chemical and Immunological Analyses of Proteins of Rat Ascites Hepatoma 682
 Hirohata, R. See *Ota, Fu and Hirohata* 532
 Hirokawa, R. See *Ogata, Omori and Hirokawa*... 660
 Horie, S. Studies on the Distribution and Phosphate-Turnover of the Acid-Soluble Phosphorus Compounds in Various Normal and Neoplastic Tissues of Rats. III. Chromatographic Isolation and Identification of Nitrogen-Containing Phosphate Esters..... 116
 Horie, S. and Shimazono, N. On the Deamination of Phosphoryl-ethanolamine in Rat Liver Homogenate..... 768
 Hosokawa, K., Nakagawa, H. and Takeda, Y. Cofactor Requirements for Anthranilate Oxidase..... 355
 Hosoya, N. and Kawada, N. Effect of Estradiol, Nucleotides and Bicarbonate on the Metabolism of Glucose and Malonate..... 728
 Huijing, F. and Slater, E.C. The Use of Oligomycin as an Inhibitor of Oxidative Phosphorylation 493

I

- Ichihara, A. and Ichihara, E.A. Metabolism of L-Lysine by Bacterial Enzymes. V. Glutaric Semialdehyde Dehydrogenase 154
 Ichihara, A. and Miyake, Y. The Activation of Glutaric Acid by an Extract from *Pseudomonas* Bacteria..... 76
 Ichihara, E.A. See *Ichihara and Ichihara* 154
 Ichihara, K. See *Tsubokura, Sakamoto and Ichihara*. 38
 Iida, K. C^{14} -Glucose Incorporation into Charoninsulfuric Acids by Slices of Mucous Gland of *Charonia lampas*..... 173
 Iijima, A. See *Hirai, Sekine, Iijima and Warabioka* 682
 Imadome, A. See *Miura, Furiya, Aso, Imadome, Nishimura and Urushibara* 519
 Imai, Y. Studies on the Gamma Ray Irradiation on Protein. I. Effect on the Sulfhydryl Groups, Polarographic Patterns and Electron Spin Resonance Absorption of Bovine Serum

- Albumin 85
 Imai, Y. Studies on the Lipogenesis in Animal Tissues under Pathological Conditions. I. The Formation of Unsaturated Fatty Acid in Diabetic and Fasted Rats 642
 Imamoto, F. and Nukada, T. On the Accumulation of Noradrenaline in Guinea Pig Brain Mitochondrial Particles 177
 Inada, Y. States of Tyrosine Residues in the Molecules of Insulin, Lysozyme and Catalase. 217
 Inoue, M. and Oikawa, A. Glycogen Phosphorylase Activities of Tumors, Regenerating Rat Liver and Suckling Rat Liver 303
 Isemura, T. See *Takagi and Isemura* 43
 Isemura, T. and Fujita-Ikeda, S. Urea Denaturation of Taka-amylase A. III. Size and Shape of Denatured Protein Molecule..... 278
 Ishibashi, I. See *Yagi and Nagatsu-Ishibashi* 441
 Ishihara, H. See *Akashi and Ishihara* 481
 Ishimoda, T. See *Hakomori and Ishimoda* 264
 —. See *Hakomori, Ishimoda, Kawauti and Eidoh*.. 307
 Ishimoto, M. and Yagi, T. Biochemical Studies on Sulfate-Reducing Bacteria. IX. Sulfite Reductase 103
 Ito, H. See *Chibata, Yamada, Ito, Furutsu and Utsumi* 373

J

- Jeuniaux, Gh., Duchâteau-Bosson, Gh. et Florkin. M. Variation de la Composante Amino-acide des Tissus et Euryhalinité chez *Perinereis cultrifera* Gr. et *Nereis diversicolor* (O.F. Müller) 527
 Jinnai, D. See *Yamamoto, Mori and Jinnai* 368

K

- Kajita, A., Hashimoto, A., Okazaki, T. and Kaziro, K. Effect of X-ray Irradiation on the Function of Hemoglobin 538
 Karazumi, I. See *Kuroda, Karazumi, Fujino, Tani, Nagai and Matsuzawa*..... 693
 Kasamaki, A. See *Sasaki, Kasamaki and Usami*... 421
 Katayama, H. See *Miura, Okamoto and Katayama* 502
 Katoh, S. Inhibitory Effect of Light on Oxygen-Uptake by Cell-Free Extracts and Particulate Fractions of *Rhodospseudomonas palustris* 126
 Kawada, M. The Quantitative Determination of Uridine Diphosphoglucuronic Acid 78
 —. See *Suzuki, Kawada and Shimazono* 448
 Kawada, N. See *Hosoya and Kawada*..... 728
 Kawai, K. Comparative Biochemical Studies on Cytochromes and Related Substances of Invertebrates. I. Cytochrome Components and Electron Transfer in Freshwater Mussels 427

K (cont'd)

- Kawai, Y., Sugita, Y. and Yoneyama, Y. Preparation of C^{14} -labeled Protoporphyrin by Hemolysed Duck Erythrocytes 80
- Kawashima, N. and Asahi, T. Sulfur Metabolism in Higher Plants. III. Further Studies on Sulfate Reduction in Excised Leaves..... 52
- Kawauti, H. See *Hakomori, Ishimoda, Kawauti and Eidoh*..... 307
- Kaziro, K. See *Kajita, Hashimoto, Okazaki and Kaziro*..... 538
- Kaziro, Y., Tanaka, R., Mano, Y. and Shimazono, N. On the Mechanism of Transpyrophosphorylation in the Biosynthesis of Thiamine Diphosphate 472
- Kikuchi, G., Abe, S. and Muto, A. Carboxylic Acids Metabolism and Its Relation to Porphyrin Biosynthesis in *Rhodopseudomonas spheroides* under Light-Anaerobic Conditions ... 570
- Kikuchi, G. and Rokugo, I. Rapid Excretion of Choleglobin in Urine after Intravenous Administration of Hemoglobin to Rabbits..... 446
- Kishimoto, Y., Takahashi, N. and Egami, F. Synthesis and Properties of Serotonin O-Sulfate 436
- Kitagawa, M., Hazama, H., Motomura, M., Yamamura, Y., Sawada, K. and Nagai, J. Chemical Studies on Cellular Components of *Hemophilus pertussis*. II. Isolation of Toxic Lipopolysaccharide..... 477
- Kitahara, K. See *Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa*..... 721
- Kobayashi, T. See *Miyamoto and Kobayashi* 548
- Kodama, K. See *Yamada, Okamoto, Kodama, Noguchi and Tanaka*..... 404
- Koike, S. See *Yamada, Koike, Yamada and Tanaka*..... 353
- Kukita, M. See *Shimizu, Maruyama, Kukita, Yanagisawa, Sato and Osaki*..... 673
- Kuroda, K., Karazumi, I., Fujino, M., Tani, Z., Nagai, A. and Matsuzawa, T. Biochemical Studies on Gingival Pigmentation in Children. 693
- Kusaka, H. See *Okumura, Kusaka and Takamatsu*. 133
- Canavanine by *Escherichia coli* 292
- Mano, Y. See *Kaziro, Tanaka, Mano and Shimazono* 472
- Mano, Y., Suzuki, K., Yamada, K. and Shimazono, N. Enzymic Studies on TPN L-Hexonate Dehydrogenase from Rat Liver ... 618
- Maruyama, Y. See *Shimizu, Maruyama, Kukita, Yanagisawa, Sato and Osaki* 673
- Masaki, Y. See *Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa* 721
- Mason, H. S. See *Anan and Mason* 765
- Masui, T. The Synthesis of Stero-Bile Acids. XXXV. Formation of Bile Acids from Cholesterol in the Organism of *Rana catesbiana* 211
- Matsubara, S. Studies on Taka-amylase A. VII. Transmaltosidation by Taka-amylase A. 226
- VIII. Influence of Substituents in Phenyl Group on the Enzymatic Hydrolysis of Phenyl- α -Maltoside by Taka-amylase A 232
- Matsumoto, M. Studies on Phospholipids. I. Comparative Analysis of Phospholipids from Mammalian Blood Stroma and Spleen ... 11
- II. Phospholipase Activity of *Clostridium perfringens* toxin 23
- III. Enzymatic Formation of Lysophosphatidylethanolamine 32
- Matsumura, Y. and Fujino, R. Radioactivation Analysis of Strontium in Rat Bone Ash..... 561
- Matsunaka, S. Studies on the Respiratory Enzyme Systems of Plants. II. The Oxidation of Reduced Diphosphopyridine Nucleotide by the Subcellular Preparations of Rice Plant Seedlings 317
- Matsuo, N. Toxicity of Cyclic Compound of β -Eleostearic Acid 635
- Matsuzawa, T. See *Kuroda, Karazumi, Fujino, Tani, Nagai and Matsuzawa* 693
- Mitsui, A., Ohta, T. and Takashima, S. Photochemical Phosphate Transfer in Green Leaves. II. Reconstruction of Reaction System 59
- Mitsui, A. and Ohta, T. Photochemical Phosphate Transfer in Green Leaves. III. Effect of Preillumination..... 65
- Mitsui, H. Incorporation of Amino Acids into Cytochrome c in a Bacterial Cell-Free System. 411
- Miura, Y., Okamoto, N. and Katayama, H. Studies on the Metabolism of Rat-Ascites-Tumor with Nitrogen Mustard Sensitive and Resistant Strains. I. On the Aerobic and Anaerobic Glycolysis 502
- Miura, Y., Okamoto, N. and Goto, M. Studies on the Metabolism of Rat-Ascites-Tumor with Nitrogen Mustard Sensitive and Resistant Strains. II. On the Oxygen Consumption and

L

- Lehninger, A. L. Inhibition of ATP-induced Contraction of Mitochondria by Polyhydroxylic Compounds 553
- Lynen, F. See *Tada und Lynen* 758

M

- Makisumi, S. Guanidino Compounds from a Sea-Anemone, *Anthopleura japonica* Verrill 284
- Decarboxylation of Hydroxyarginine and

M (cont'd)

Oxidative Phosphorylation	508
Miura, Y., Momose, K. and Moriyama, A. Studies on the Metabolism of Rat-Ascites-Tumor with Nitrogen Mustard Sensitive and Resistant Strains. III. On the Incorporation of Labelled Amino Acids into Protein <i>in vitro</i> ...	514
Miura, Y., Furiya, S., Aso, Y., Imadome, A., Nishimura, A. and Urushibara, O. Studies on the Metabolism of Rat-Ascites-Tumor with Nitrogen Mustard Sensitive and Resistant Strains. IV. On the Distribution of Various Enzymes in the Cells of Normal Liver, Regenerating Liver and Hepatoma	519
Miyake, Y. See <i>Ichihara and Miyake</i>	76
Miyamoto, S. and Kobayashi, T. High-Voltage Paper Electrophoresis of Iodine-Containing Compounds in Thyroid Gland	548
Momose, K. See <i>Miura, Momose and Moriyama</i> . ..	514
Mori, A. See <i>Yamamoto, Mori and Jinnai</i>	368
Morimoto, S. and Yoneyama, Y. Fate of Radioactive Tin in Rats	601
Morino, Y. See <i>Fujioka, Morino and Sakamoto</i> ...	333
— See <i>Nakahara, Morino, Morisue and Sakamoto</i> . ..	339
— See <i>Nakahara, Watanabe, Morino and Sakamoto</i> . ..	343
Morisue, T. See <i>Nakahara, Morino, Morisue and Sakamoto</i>	339
Moriyama, A. See <i>Miura, Momose and Moriyama</i> . ..	514
Motomura, M. See <i>Kitagawa, Hazama, Motomura, Yamamura, Sawada and Nagai</i>	477
Muramatsu, M., Nagayama, H. and Shimura, K. Studies on the Biosynthesis of Glycine in the Silkworm. I. Formation of Glycine from Serine	55
Muto, A. See <i>Kikuchi, Abe and Muto</i>	570

N

Nagai, A. See <i>Kuroda, Karazumi, Fujino, Tani, Nagai and Matsuzawa</i>	693
Nagai, J. See <i>Kitagawa, Hazama, Motomura, Yamamura, Sawada and Nagai</i>	477
Nagatsu-Ishibashi, I. See <i>Yagi and Nagatsu-Ishibashi</i>	441
Nagayama, H. See <i>Muramatsu, Nagayama and Shimura</i>	55
Nagayama, R. See <i>Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa</i>	721
Nagumo, T. Complexes between Acidic and Basic Proteins	379
Nakagawa, H. See <i>Hosokawa, Nakagawa and Takeda</i>	355
Nakagawa, H., Suzuki, F. and Takeda, Y. Studies on Formate Activation. I. Purification and Properties of Formokinase	191

Nakahara, I., Morino, Y., Morisue, T. and Sakamoto, Y. Enzymatic Studies on Pyridoxine Metabolism. VI. Pyridoxine Metabolism in Pregnancy.....	339
Nakahara, I., Watanabe, Y., Morino, Y. and Sakamoto, Y. Enzymatic Studies on Pyridoxine Metabolism. VII. The Influence of Fat and Fatty Acid Administration upon Pyridoxine Metabolism	343
Nakamura, S. and Wakeyama, T. An Attempt to Demonstrate the Distribution of Trypsin Inhibitors in the Sera of Various Animals.....	733
Nakao, M. See <i>Hashimoto, Nakao and Yoshikawa</i> . ..	595
Nakao, M., Nakao, T., Yamazoe, S. and Yoshikawa, H. Adenosine Triphosphate and Shape of Erythrocytes	487
Nakao, T. See <i>Nakao, Nakao, Yamazoe and Yoshikawa</i>	487
Nakatani, M. Studies on Histidine Residues in Hemeprotein Related to Their Activities. V. Photooxidation of Catalase in the Presence of Methylene Blue	98
Nakayama, S. Studies on the Denaturation of Enzymes. III. Effects of Anions on Heat Inactivation of Sweet Potato β -Amylase	328
Nakayama, T. Studies on Acetic Acid Bacteria. III. Purification and Properties of Coenzyme-Independent Aldehyde Dehydrogenase	158
— IV. Purification and Properties of a New Type of Alcohol Dehydrogenase, Alcohol-Cytochrome-553 Reductase	240
Nishida, M. Action of Pancreatic Lipase in Homogeneous Solution. IV. Effect of Synthetic Food Colors on Pancreatic Lipase	169
Nishimura, A. See <i>Miura, Furiya, Aso, Imadome, Nishimura and Urushibara</i>	519
Noguchi, F. See <i>Yamada, Okamoto, Kodama, Noguchi and Tanaka</i>	404
Nukada, T. See <i>Imamoto and Nukada</i>	177

O

Oda, T. See <i>Green and Oda</i>	742
Ogata, K., Omori, S., Hirokawa, R. Studies on the Biosynthesis of Antibody and Other γ -Globulin in Microsomes of Immunized Spleen Cells	660
Ogura, M. and Yamasaki, K. The Fate of C^{14} -Labeled Dehydrocholic Acid in Guinea Pig. ..	649
Ohnishi, T. See <i>Ohnishi and Ohnishi</i>	83
Ohnishi, T. and Ohnishi, T. Application of Hexanol-celite Column for an Assay of Inorganic Phosphate	83
Ohta, T. See <i>Mitsui, Ohta and Takashima</i>	59
— See <i>Mitsui and Ohta</i>	65

O (cont'd)

Oikawa, A. See <i>Inoue and Oikawa</i>	303	Sawada, F. See <i>Ando and Sawada</i>	252
Okamoto, K. See <i>Yamada, Okamoto, Kodama, Noguchi and Tanaka</i>	404	Sawada, K. See <i>Kitagawa, Hazama, Motomura, Yamamura, Sawada and Nagai</i>	477
Okamoto, N. See <i>Miura, Okamoto and Katayama</i>	502	Seiji, M. and Fitzpatrick, T. B. The Reciprocal Relationship between Melanization and Tyrosinase Activity in Melanosomes (Melanin Granules)	700
Okamoto, N. See <i>Miura, Okamoto and Goto</i>	508	Sekine, K. See <i>Hirai, Sekine, Iijima and Warabioka</i>	682
Okazaki, T. See <i>Kajita, Hashimoto, Okazaki and Kaziro</i>	538	Shibata, N., Yamashita, S. and Yasuda, K. On the Minimum Requirement and Treatment Dosis of Thiamine for Rats.....	589
Okumura, F. S., Kusaka, H. and Takamatsu, T. Studies on Isokinetin and Its Analogues. I. Synthesis of Isokinetin, 2-N-Furfurylamino-purine, and Its Activity of Promoting Growth of Leaves	133	Shiio, I., Ôtsuka, S. and Takahashi, M. Effect of Growth Substrates on Levels of Glyoxylate By-pass Enzymes in a Glutamate Forming Bacterium, <i>Brevibacterium flavum</i> No. 2247	262
Okunuki, K. See <i>Yamanaka, Ota and Okunuki</i> ..	414	— Glutamic Acid Formation from Glucose by Bacteria. V. Oxidative Metabolism of Glucose in <i>Brevibacterium flavum</i> No. 2247	397
Omori, S. See <i>Ogata, Omori and Hirokawa</i>	660	Shiio, I. and Tsunoda, T. Bacterial Formation of Glutamic Acid from Acetic Acid.*	
Osaki, S. See <i>Shimizu, Maruyama, Kukita, Yanagisawa, Sato and Osaki</i>	673	II. Formation of Glutamic Acid from C ¹⁴ -Labelled Acetic Acid in <i>Brevibacterium flavum</i> , No. 2247	141
Ota, A. See <i>Yamanaka, Ota and Okunuki</i>	414	— III. Incorporation of Labelled Acetate and Kinetic Relationships of the Metabolites in <i>Brevibacterium flavum</i> , No. 2247.	148
Ota, S., Fu, T. H. and Hirohata, R. Studies on Bromelain. II. Its Activation and Fractionation	532	Shimao, K. The Estimation of Serum Proteins by Ultraviolet Densitometry of Agar Electrophoretic Diagram	451
Ôtsuka, S. See <i>Shiio, Ôtsuka and Takahashi</i> ..	262, 379	Shimazono, N. See <i>Suzuki, Kawada and Shimazono</i>	448
R		— See <i>Horie and Shimazono</i>	451
Rimington, C. and Tooth, B. E. Role of Mitochondria in the <i>in vitro</i> Formation of Protoporphyrin and Haem	456	— See <i>Kaziro, Tanaka, Mano and Shimazono</i>	472
Rokugo, I. See <i>Kikuchi and Rokugo</i>	446	— See <i>Mano, Suzuki, Yamada and Shimazono</i>	618
S		Shimizu, M., Maruyama, Y., Kukita, M., Yanagisawa, Y., Sato, T. and Osaki, S. On the Determination of Serum Caeruloplasmin, and the Results of Its Measurement	673
Sagisaka, S. and Shimura, K. Studies in Lysine Biosynthesis. II. Metabolic Fate of DL- α -Aminoadipic Acid-6-C ¹⁴ in <i>T. utilis</i>	392	Shimura, K. See <i>Muramatsu, Nagayama and Shimura</i>	55
Sakamoto, Y. See <i>Tsubokura, Sakamoto and Ichihara</i>	38	— See <i>Suto and Shimura</i>	69
— See <i>Fujioka, Morino and Sakamoto</i>	333	— See <i>Suzuka, Tanaka and Shimura</i>	81
— See <i>Nakahara, Morino, Morisue and Sakamoto</i>	339	— See <i>Soejima and Shimura</i>	260
— See <i>Nakahara, Watanabe, Marino and Sakamoto</i>	343	— See <i>Sagisaka and Shimura</i>	392
Sakurada, T. Dephosphorylative Decomposition of Phosphagen (Phosphocreatine) by Zirconyl Ion	91	Shinoda, S. Über das Vorkommen der Freien Aminosäuren in Verschiedenen Rinderorganen.	186
Sasakawa, S. Studies on Hemoglobin. V. Chromatographic Resolution of Bovine Globin into the Valylleucyl and Methionyl Chains.....	200	Slater, E. C. See <i>Huijing and Slater</i>	493
Sasaki, S., Kasamaki, A. and Usami, S. On the Mechanism of Anaerobic Formation of Succinate by <i>Proteus vulgaris</i>	421	Soejima, M. and Shimura, K. Preparation of Reduced, Activator-Free Papain by Para-Thiocresol Treatment.....	260
Sato, T. See <i>Shimizu, Maruyama, Kukita, Yanagisawa, Sato and Osaki</i>	673	Sugai, S. Studies of Streptolysin S' Formation by Streptococcal Ghosts.....	348
Sato, T., Fukuyama, T., Suzuki, T., Yoshikawa, H. and Gorrod, J. W. Studies on Conjugation of S ³⁵ -Sulfate with Phenolic Compounds. VI. Sulfate Conjugation of Xanthurenic Acid in Rat Liver	164	Sugita, Y. See <i>Kawai, Sugita and Yoneyama</i>	80

S (cont'd)

- Suto, S. and Shimura, K. Studies on the Biosynthesis of Silk Fibroin. III. *In vivo* incorporation of Glycine- C^{14} into Proteins of Posterior Silk gland Fractions..... 69
- Suzuka, I., Tanaka, S. and Shimura, K. Amino Acid Activating Enzyme Free from Pyrophosphate Exchange 81
- Suzuki, F. See *Nakagawa, Suzuki and Takeda*.... 191
- Suzuki, F. and Takeda, Y. Synthesis of Formyl Phosphate 49
- Suzuki, K. See *Mano, Suzuki, Yamada and Shimazono* 618
- Suzuki, K., Kawada, M. and Shimazono, N. Further Studies on Soluble Lactonase; Identity of Lactonase I and Aldonolactonase with Gluconolactonase 448
- Suzuki, T. See *Sato, Fukuyama, Suzuki, Yoshikawa and Gorrod*..... 164

T

- Tada, M. und Lynen, F. Zur Biosynthese der Terpene. XIV Zur Bestimmung von Phosphomevalonsäure-Kinase und Pyrophosphomevalonsäure-Decarboxylase in Zellextrakten.... 758
- Takagi, T. and Isemura, T. Studies on the Amphoteric Properties of Taka-amylase A. II. Unmasking of Acid Binding Sites..... 43
- Takahashi, K. The Structure and Function of Ribonuclease T_1 . I. Chromatographic Purification and Properties of Ribonuclease T_1 1
- Takahashi, M. See *Shiio, Ôtsuka and Takahashi*.. 262, 397
- Takahashi, N. See *Kishimoto, Takahashi and Egami*..... 436
- Takahashi, N. and Egami, F. Steroidsulfatase in the Liver of *Charonia lampas* 358
- Takahashi, Y. and Tanaka, K. Gas-chromatographic Analysis of the Fatty Acid Composition of Human Fatty Liver..... 713
- Takamatsu, T. See *Okumura, Kusaka and Takamatsu* 133
- Takashima, S. See *Mitsui, Ohta and Takashima*. 59
- Take, T. On the Dissociation of Hemoglobin under the Action of Urea 206
- Takeda, Y. See *Suzuki and Takeda*..... 49
- See *Nakagawa, Suzuki and Takeda*..... 191
- See *Hosokawa, Nakagawa and Takeda* 355
- Takeyama, S. and Buchanan, J. M. Enzymatic Synthesis of the Methyl Group of Methionine. III. Spectral and Electrophoretic Studies of the Prosthetic Group of the B_{12} Enzyme..... 578
- Tanaka, K. See *Takahashi and Tanaka* 713
- Tanaka, R. *Kaziro, Tanaka, Mano and Shimazono*. 472
- Tanaka, S. See *Suzuka, Tanaka and Shimura*..... 81

- Tanaka, S. See *Yamada, Koike, Yamada and Tanaka*..... 353
- See *Yamada, Okamoto, Kodama, Noguchi and Tanaka*..... 404
- Tani, Z. See *Kuroda, Karazumi, Fujino, Tani, Nagai and Matsuzawa* 693
- Tarutani, O. See *Ui and Tarutani*..... 9
- Tokuyasu, K. and Funatsu, M. Studies on Enzymatically Active Fragments of Pepsin. II. Isolation and Purification of Fragments with Peptic Activity..... 297
- Tooth, B. E. See *Rimington and Tooth*..... 456
- Tsubokura, S., Sakamoto, Y. and Ichihara, K. The Bacterial Decomposition of Indoleacetic Acid 38
- Tsukamura, M. A Labile Phosphorus Compound Showing Phosphorus Turnover Appearing in Protein Fraction of a *Mycobacterium*..... 138
- Tsunoda, T. See *Shiio and Tsunoda*..... 141, 148

U

- Ui, N. and Tarutani, O. Addendum: Sedimentation and Diffusion of Ribonuclease T_1 9
- Urushibara, O. See *Miura, Furiya, Aso, Imadome, Nishimura and Urushibara* 519
- Usami, S. See *Sasaki, Kasamaki and Usami*..... 421
- Utsumi, I. See *Chibata, Yamada, Ito, Furutsu and Utsumi*..... 373

W

- Wakeyama, T. See *Nakamura and Wakeyama*..... 733
- Warabioka, K. See *Hirai, Sekine, Iijima and Warabioka* 682
- Watanabe, T. See *Akamatsu and Watanabe*..... 566
- Watanabe, Y. See *Nakahara, Watanabe, Morino and Sakamoto*..... 343
- Wiss, O. und Brubacher, G. Zur Beeinflussung des Ubichinongehaltes der Leber wachsender Ratten durch verschiedene Diätformen 609

Y

- Yagi, K. and Nagatsu-Ishibashi, I. Intracellular Distribution of D-Amino Acid Oxidase in Rat Liver..... 441
- Yagi, M. See *Higashi, Yagi and Hirai* 707
- Yagi, T. See *Ishimoto and Yagi* 103
- Yamada, H., Koike, S., Yamada, K. and Tanaka, S. An Improved Method for the Preparation of Triphosphopyridine Nucleotide from Pig Liver..... 353
- Yamada, H., Okamoto, K., Kodama, K., Noguchi, F. and Tanaka, S. Enzymic Studies on Mannitol Formation by *Piricularia oryzae*... 404
- Yamada, K. See *Yamada, Koike, Yamada and*

Y (cont'd)		Catalase.....	414
<i>Tanaka</i>	353	Yamasaki, K. See <i>Ogura and Yamasaki</i>	649
Yamada, K. See <i>Mano, Suzuki, Yamada and Shimazono</i>	618	Yamashita, S. See <i>Shibata, Yamashita and Yasuda</i> ..	589
Yamada, S. See <i>Ghibata, Yamada, Ito, Furutsu and Utsumi</i>	373	Yamazoe, S. See <i>Nakao, Nakao, Yamazoe and Yoshikawa</i>	487
Yamamoto, Y., Mori, A. and Jinnai, D. Amino Acids in the Brain. Alteration of Amino Acids in Rabbit Brain caused by Repetitive Convulsive Fits and Comparison of Amino Acid Contents in Epileptic and Non-epileptic Human Brain.....	368	Yanagisawa, I. See <i>Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa</i>	721
Yamamura, Y. See <i>Kitagawa, Hazama, Motomura, Yamamura, Sawada and Naga i</i>	477	Yanagisawa, Y. See <i>Shimizu, Maruyama, Kukita, Yanagisawa, Sato and Osaki</i>	673
Yamanaka, T., Ota, A. and Okunuki, K. Activation of <i>Pseudomonas</i> Cytochrome Oxidase by		Yasuda, K. See <i>Shibata, Yamashita and Yasuda</i> ..	589
		Yoneyama, Y. See <i>Kawai, Sugita and Yoneyama</i> ..	80
		—, See <i>Morimoto and Yoneyama</i>	601
		Yoshikawa, H. See <i>Sato, Fukuyama, Suzuki, Yoshikawa and Gorrod</i>	164
		—, See <i>Nakao, Nakao, Yamazoe and Yoshikawa</i> ..	487
		—, See <i>Hashimoto, Nakao and Yoshikawa</i>	595

INDEX TO SUBJECTS

A

Acetate: Incorporation of labelled —, Bacterial formation, Glutamic acid, <i>Brevibacterium flavum</i> No. 2247.....	148
Acetic acid: C ¹⁴ -labelled — —, Bacterial formation, Glutamic acid, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio and Tsunoda</i>	141
— —: — — bacteria, Coenzyme-independent, Aldehyde, Dehydrogenase, <i>Nakayama</i>	158
— —: — — —; Alcohol, Dehydrogenase, Alcohol-cytochrome-553, Reductase, <i>Nakayama</i>	240
Acidic: — Basic, Proteins complex, <i>Nagumo</i>	379
Activation: <i>Pseudomonas</i> , Cytochrome Oxidase, Catalase, <i>Yamanaka, Ota and Okunuki</i>	414
—: Bromelain, Fractionation, <i>Ota, Fu and Hirohata</i>	532
—: Muscle Glycerate, 2, 3-Diphosphatase, Mercuric ion, <i>Hashimoto, Nakao and Yoshikawa</i>	595
Activity: Tyrosinase —, Melanization, Melanosome(s) (Melanin Granules), <i>Seiji and Fitzpatrick</i>	700
Adenosine triphosphate: Erythrocyte, <i>Nakao, Nakao, Yamazoe and Yoshikawa</i>	487
Aerobic: — and Anaerobic Glycolysis, Metabolism, Rat-Ascites-tumor, Nitrogen mustard, Strain, <i>Miura, Okamoto and Katayama</i>	502
Agar: — Electrophoretic diagram, Serum Proteins, Ultraviolet Densitometry, <i>Shimao</i>	451
Alanine: Vitamin B ₆ , Lactic acid bacteria, <i>Chibata, Yamada, Ito, Furutsu and Utsumi</i>	373
Albumin: Bovine serum —, gamma ray irradiation, Protein, sulfhydryl group, polarographic pattern, electron spin resonance absorption, <i>Imai</i>	85
Alcohol: — Dehydrogenase, —cytochrome-553 reductase, Acetic acid bacteria, <i>Nakayama</i>	240
Aldehyde: Coenzyme-independent — dehydrogenase, Acetic acid bacteria, <i>Nakayama</i>	158
Aldonolactonase: Lactonase, Soluble —, — I, Gluconolactonase, <i>Suzuki, Kawada and Shimazono</i>	448
Alkaline treatment: Amino acid, Yeast, RNA, <i>Akashi and Ishihara</i>	481
Amino acid: — — activating enzyme, <i>Suzuka, Tanaka and Shimura</i>	81
—: Alteration of —, Comparison of — contents, Brain, Convulsive fits, Epileptic, <i>Yamamoto, Mori and Jinnai</i>	368
—: Incorporation, Cytochrome c, Bacterial,	

Cell-free system, <i>Mitsui</i>	411
D—: — Oxidase, Intracellular, Liver, <i>Yagi and Nagatsu-Ishibashi</i>	441
—: liveration of —, Yeast, RNA, Alkaline treatment, <i>Akashi and Ishihara</i>	481
—: metabolism, Rat-Ascites-tumor, Nitrogen mustard, Strain, Incorporation, Protein, <i>In vitro</i> , <i>Miura, Momose and Moriyama</i>	514
—: euryhalinité, <i>Perinereis cultrifera</i> Gr. <i>Nereis diversicolor</i> O. F. Müller, <i>Jeuniaux, Duchâteau-Bosson et Florkin</i>	527
DL- α -Aminoadipic acid-6-C ¹⁴ : Lysine Biosynthesis, Metabolic, <i>T. utilis</i> , <i>Sagisaka and Shimura</i>	392
Aminosäure: Vorkommen der freien —, <i>Shinoda</i>	186
β -Amylase: Sweet potato —, <i>Nakayama</i>	328
Anaerobic: — formation, Mechanism, <i>Sasaki, Kasamaki and Usami</i>	421
—: Aerobic and — Glycolysis, metabolism, Rat-Ascites-tumor, Nitrogen mustard, Strain, <i>Miura, Okamoto and Katayama</i>	502
—: Light- — conditions, Carboxylic acids Metabolism, Porphyrin Biosynthesis, <i>Rhodopseudomonas spheroides</i> , <i>Kikuchi, Abe and Muto</i>	570
Analysis: Radioactivation —, Strontium, Bone ash, <i>Matsumura and Fujino</i>	561
<i>Anthopleura japonica</i> Verril: Sea-anemone, Guanidino compound, <i>Makisumi</i>	284
Anthranilate: —, Oxidase, Cofactor requirement, <i>Hosokawa, Nakagawa and Takeda</i>	355
Antibody: Biosynthesis, γ -globulin, Microsome, Immunized Spleen Cell, <i>Ogata, Omori and Hirokawa</i>	660
Arginase: γ -Hydroxyarginine, Guanidino, Sea-cucumber, Arginine Decarboxylase, <i>Fujita</i>	468
Arginine: — Decarboxylase, γ -Hydroxyarginine, Guanidino, Sea-cucumber, Arginase, <i>Fujita</i>	468
—: Determination, <i>Akamatsu and Watanabe</i>	566
Ascites Hepatoma: See <i>Rat Ascites Hepatoma</i>	
Ascites-tumor: See <i>Rat-Ascites tumor</i>	
ATP: —-induced Contraction, Inhibition, Mitochondria, Polyhydroxylic compounds, <i>Lehninger</i>	553

B

Bacteria: Sulfate-reducing —, sulfite reductase, <i>Ishimoto and Yagi</i>	103
—: Acetic acid —, Coenzyme-independent, Aldehyde, Dehydrogenase, <i>Nakayama</i>	158
—: Acetic acid —, Alcohol Dehydrogenase,	

B (Cont'd)

Alcohol-cytochrome-553 reductase, <i>Nakayama</i> ...	240
—: Lactic acid —, Alanine, Vitamin B ₆ , <i>Chibata, Yamada, Ito, Furutsu and Utsumi</i>	373
—: Glutamic acid, Glucose, Oxidative Metabolism, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio, Ôtsuka and Takahashi</i>	397
Bacterial: — Cell-free system, Incorporation, Amino acid, Cytochrome c, <i>Mitui</i>	411
Bacterium: — al decomposition, Indoleacetic acid, <i>Tsubokura, Sakamoto and Ichihara</i>	38
—: —al formation, Glutamic acid, C ¹⁴ -labelled acetic acid, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio and Tsunoda</i>	141
—: —al formation Glutamic acid, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio and Tsunoda</i>	148
—: Glutamate forming —, <i>Brevibacterium flavum</i> , No. 2247, Glyoxylate by-pass enzyme, <i>Shiio, Ôtsuka and Takahashi</i>	262
Basic: Acidic and — proteins, <i>Nagumo</i>	379
Bicarbonate: Estradiol, Nucleotide(s), Metabolism, Glucose, Malonate, <i>Hosoya and Kawada</i> ...	728
Bile acid: Formation of —, Cholesterol, <i>Rana catesbiana</i> Stero bile acid, <i>Masui</i>	211
Biosynthesis: Phosphomevalonsäure-Kinase, Pyrophosphomevalonsäure-Decarboxylase, Terpene XIV, <i>Tada und Lynen</i>	758
Biosynthesis: Lysine —, DL- α -Aminoadipic acid-6-C ¹⁴ , <i>T. utilis</i> , <i>Sagisaka and Shimura</i>	392
—: Isovalthine, <i>Fukutome</i>	444
—: — of Thiamine diphosphate, Mechanism, Transpyrophosphorylation, <i>Kaziro, Tanaka, Mano and Shimazono</i>	472
—: Porphyrin —, Carboxylic acids metabolism, <i>Rhodopseudomonas spheroides</i> Light-Anaerobic, <i>Kikuchi, Abe and Muto</i>	570
—: Antibody, γ -Globulin, Microsome, Immunized Spleen Cell, <i>Ogata, Omori and Hirokawa</i>	660
Blood stroma: Mammalian — —, Phospholipid, <i>Matsumoto</i>	11
Bone ash: Radioactivation Analysis, Strontium, <i>Matsumura and Fujino</i>	561
Brain: epileptic and non-epileptic human Brain, Amino acid, Convulsive fits, <i>Yamamoto, Mori and Jinnai</i>	368
<i>Brevibacterium flavum</i> No. 2247: Bacterial formation, Glutamic acid, C ¹⁴ -labelled acetic acid, <i>Shiio and Tsunoda</i>	141
—: Bacterial formation, Glutamic acid, <i>Shiio and Tsunoda</i>	148
—: Glutamate forming bacterium, Glyoxylate by-pass enzyme, <i>Shiio, Ôtsuka and Takahashi</i> ...	262
—: Glutamic acid, Glucose, Bacteria, Oxidative	

Metabolism, <i>Shiio, Ôtsuka and Takahashi</i>	397
α -Bromoisovalerylurea: (Bromural), Sedative, <i>Haruna</i>	388
Bromelain: Activation, Fractionation, <i>Ota, Fu and Hirohata</i>	532
Bromural: α -Bromoisovalerylurea, Sedative, <i>Haruna</i>	388

C

C ¹⁴ : Glycine—, <i>Suto and Shimura</i>	69
—: —labelled, Protoporphyrin, Erythrocytes, <i>Kawai, Sugita and Yoneyama</i>	80
—: Acetic acid, Bacterial formation Glutamic acid, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio and Tsunoda</i>	141
—: —Glucose incorporation, <i>Charonia lampas</i> , <i>Iida</i>	173
Caeruloplasmin: Serum —, Determination, <i>Shimizu, Maruyama, Kukita, Yanagisawa, Sato and Osaki</i>	673
Canavanine: Decarboxylation, Hydroxyarginine, <i>Escherichia coli</i> , <i>Makisumi</i>	292
Cancer: Abnormal γ -Glycoprotein, <i>Hakomori, Ishimoda, Kawauti and Eidoh</i>	307
Carboxylic acid: —s Metabolism, Porphyrin Biosynthesis, <i>Rhodopseudomonas spheroides</i> , Light-Anaerobic, <i>Kikuchi, Abe and Muto</i>	570
Catalase: Photooxidation of —, Histidine residue, Hemeprotein, <i>Nakatani</i>	98
—: Tyrosine residue, Insulin, Lysozyme, <i>Inada</i>	217
—: Activation, <i>Pseudomonas</i> , Cytochrome Oxidase, <i>Yamanaka, Ota and Okunuki</i>	414
—: Immunochemical, Erythrocyte(s), Liver, <i>Higashi, Yagi and Hirai</i>	707
Cell: —free system, Incorporation, Amino acid, Cytochrome c, Bacterial, <i>Mitui</i>	411
—: Metabolism, Rat-Ascites-tumor, Enzyme, Liver, Hepatoma, <i>Miura, Furiya, Aso, Imadome, Nishimura and Urushibara</i>	519
—: Immunized Spleen —, Biosynthesis, Antibody, γ -globulin, Microsome, <i>Ogata, Omori and Hirokawa</i>	660
Cellular components: <i>Hemophilus pertussis</i> , Isolation, Toxic Lipopolysaccharide, <i>Kitagawa, Hazama, Motomura, Yamamura, Sawada and Nagai</i>	477
<i>Charonia lampas</i> : C ¹⁴ -glucose incorporation into Charonin-sulfuric acid, <i>Iida</i>	173
—: Steroidsulfatase, <i>Takahashi and Egami</i>	358
Choleglobin: excretion of —, Urine, Hemoglobin, <i>Kikuchi and Rokugo</i>	446
Cholesterol: Formation of bile acid, <i>Ranacatesbiana</i> , synthesis of stero-bile acid, <i>Masui</i>	211
Chromatography: —ic purification, properties	

C (Cont'd)

- of ribonuclease T₁, *Takahashi*..... 1
- : —ic isolation and identification of nitrogen-containing phosphate ester, phosphate-turnover, acid soluble phosphorus compound in neoplastic tissue, *Horie* 116
- : —ic resolution, bovine globulin, valylleucyl and methionyl chain, *Sasakawa*..... 200
- : Gas- —ic analysis, Fatty acid, Fatty liver, *Takahashi and Tanaka*..... 713
- : DEAE-cellulose —, Conversion, Prothrombin, Thrombin, Isolation, *Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa*... 721
- Chromoprotein: *Nori, Porphyra tenera*, Sugar component, Phycoerythrin, *Fujiwara*..... 361
- Glostridium perfringens*: — — toxin, phospholipase activity, phospholipid, *Matsumoto*..... 23
- Clupeine: Fractionation, protamine, *Ando and Sawada*..... 252
- Coenzyme: — -independent aldehyde dehydrogenase, acetic acid bacteria, *Nakayama*..... 158
- Cofactor: Requirement, anthranilate oxidase, *Hosokawa, Nakagawa and Takeda* 355
- Comparative biochemical: — — studies, Cytochrome, Invertebrate, Electron transfer, Fresh-water mussels, *Kawai*..... 427
- Contraction: ATP-induced —, Inhibition, Mitochondria, Polyhydroxylic compounds, *Lehninger*. 553
- Conversion: Prothrombin, Thrombin, DEAE-cellulose chromatography, Isolation, *Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa*..... 721
- Convulsive fits: Amino acid, Brain, Epileptic, *Yamamoto, Mori and Jinnai* 368
- Cyclic: — compound, Oil(s), Fat(s), Toxicity, β -Eleostearic acid, *Matsuo*..... 635
- Cytochrome: Alcohol- — -553 reductase, Alcohol dehydrogenase, Acetic acid bacteria, *Nakayama*. 240
- c: Incorporation, Amino acid, Bacterial, Cell-free system, *Mitsui*..... 411
- : — Oxidase, Activation, *Pseudomonas*, Catalase, *Yamanaka, Ota and Okunuki* 414
- : Comparative biochemical, Invertebrate, Electron transfer, Fresh-water mussels, *Kawai*. 427

D

- DEAE-cellulose: — Chromatography; Conversion, Thrombin, Prothrombin, Isolation, *Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa*..... 721
- Decarboxylase: arginine —, γ -Hydroxyarginine, Guanidino, Sea-cucumber, Arginase, *Fujita*.... 468
- Decarboxylation: — of Hydroxyarginine, Canavanine, *Escherichia coli*, *Makisumi*..... 292

- Dehydrocholic acid: C¹⁴-Labeled —, *Ogura and Yamasaki* 649
- Dehydrogenase: Glutaric semialdehyde —, Bacterial enzyme, L-lysine, *Ichihara and Ichihara*... 154
- : Coenzyme-independent, Aldehyde —, Acetic acid bacteria, *Nakayama*..... 158
- : Alcohol —, Alcohol-cytochrome-553 reductase, Acetic acid bacteria, *Nakayama*..... 240
- : TPN L-Hexonate —, Enzymic, Liver, *Mano, Suzuki, Yamada and Shimazono* 618
- Densitometry: Ultraviolet —, Serum Proteins, Agar Electrophoretic, *Shimao*..... 451
- Dephosphorylation: —ive decomposition, Phosphagen, Phosphocreatine, Zirconyl ion, *Sakurada*..... 91
- Determination: Arginine, *Akamatsu and Watanabe*..... 566
- : Serum Caeruloplasmin, *Shimizu, Maruyama, Kukita, Yanagisawa, Sato and Osaki*..... 673
- Diabetic: — and fasted rats, Lipogenesis, Pathological, unsaturated Fatty acid, *Imai*..... 642
- Diätformen: Ubichinon, Leber, *Wiss und Brubacher*..... 609
- Diphosphopyridine nucleotide: Oxidation, *Matsunaka*..... 317
- DPN: See *Diphosphopyridine nucleotide*

E

- Electron spin resonance absorption: Gamma ray, Protein, sulfhydryl group, Polarographic pattern, Serum albumin, *Imai*..... 85
- Electron transfer: Comparative biochemical, Cytochrome, Invertebrate, Fresh-water mussels, *Kawai*..... 427
- Electrophoresis: High-voltage paper —, Iodine, Thyroid gland, *Miyamoto and Kobayashi* 548
- Electrophoretic: Agar — diagram, Serum Proteins, Ultraviolet Densitometry, *Shimao*..... 451
- : Enzymatic Synthesis, Methyl group, Methionine, Spectral, Prosthetic group, B₁₂ Enzyme, *Takeyama and Buchanan* 578
- β -Eleostearic acid: Oil(s), Fat(s), Toxicity, Cyclic, *Matsuo*..... 635
- Enzymatic: — Synthesis, methyl group, methionine, spectral, Electrophoretic, Prosthetic group, B₁₂ Enzyme, *Takeyama and Buchanan*..... 578
- Enzyme: —atic formation, Lysophosphatidylethanol-amine, Phospholipid, *Matsumoto* 32
- : Amino acid activating —, *Suzuka, Tanaka and Shimura*..... 81
- : Bacterial —, L-lysine, Glutaric semialdehyde dehydrogenase, *Ichihara and Ichihara*..... 154
- : —atic hydrolysis, Phenyl- α -maltoside, Taka-amylase A, *Matsubara*..... 232

E (Cont'd)

—: Glyoxylate by-pass —, Glutamate forming bacterium, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio, Ôtsuka and Takahashi</i>	262
—: Respiratory —, Oxidation of reduced diphosphopyridine nucleotide, <i>Matsunaka</i>	317
—: denaturation of —, β -Amylase, <i>Nakayama</i> ...	328
—: —atic, Reduction, Isopyridoxal, Pyridoxine metabolism, <i>Fujioka, Morino and Sakamoto</i>	333
—: metabolism, Rat-Ascites-tumor, Cell, Liver, Hepatoma, <i>Miura, Furiya, Aso, Imadome, Nishimura and Urushibara</i>	519
B ₁₂ —: Enzymatic Synthesis, Methyl group, Methionine, Spectral, Electrophoretic, Prosthetic group, <i>Takeyama and Buchanan</i>	578
Enzymic: — Studies, Mannitol, <i>Piricularia oryzae</i> , <i>Yamada, Okamoto, Kodama, Noguchi and Tanaka</i>	404
—: — Studies, TPN L-Hexonate Dehydrogenase, Liver, <i>Mano, Suzuki, Yamada and Shimazono</i>	618
Epileptic: — and Non-epileptic Brain, Amino acid, Brain, Convulsive fits, <i>Yamamoto, Mori and Jinmai</i>	368
Erythrocyte: hemolysed duck —, Protoporphyrin, <i>Kawai, Sugita and Yoneyama</i>	80
—: Adenosine triphosphate, <i>Nakao, Nakao, Yamazoe and Yoshikawa</i>	487
—: Immunochemical, Catalase, Liver, <i>Higashi, Yagi and Hirai</i>	707
<i>Escherichia coli</i> : Decarboxylation, Hydroxyarginine, Canavanine, <i>Makisumi</i>	292
Estradiol: Nucleotide(s), Bicarbonate, Metabolism, Glucose, Malonate, <i>Hosoya and Kawada</i>	728
Euryhalinité: Amino-acide, <i>Perinereis cultrifera</i> Gr. <i>Nereis diversicolor</i> O.F. Müller, <i>Jeuniaux, Duchâteau-Bosson et Florkin</i>	527

F

Fat: Fatty acid, Pyridoxine metabolism, <i>Nakahara, Watanabe, Morino and Sakamoto</i>	343
Fatty acid: Fatt, Pyridoxine metabolism, <i>Nakahara, Watanabe, Morino and Sakamoto</i>	343
—: Unsaturated —, Lipogenesis, pathological, Diabetic, <i>Imai</i>	642
—: Gas-Chromatographic, Fatty liver, <i>Takahashi and Tanaka</i>	713
Fatty liver: Gas-Chromatographic, Fatty acid, <i>Takahashi and Tanaka</i>	713
Fibroin: Silk —, glycine-C ¹⁴ , <i>Suto and Shimura</i>	69
Formate: — activation, Formokinase, <i>Nakagawa, Suzuki and Takeda</i>	191
Formokinase: Formate activation, <i>Nakagawa, Suzuki and Takeda</i>	191
Formyl-hexosamine: N- —, Glycoprotein,	

<i>Hakomori and Ishimoda</i>	264
Formyl phosphate: <i>Suzuki and Takeda</i>	49
Formyl-residue: N-formylhexosamine, <i>Hakomori and Ishimoda</i>	264
Fractionation: Bromelain, Activation, <i>Ota, Fu and Hirohata</i>	532
Fresh-water mussels: Comparative biochemical, Cytochrome, Invertebrate, Electron transfer, <i>Kawai</i>	427
Furfurylaminopurine: 2-N- —, Isokinetine, <i>Okumura, Kusaka and Takamatsu</i>	133

G

Gamma Ray: — — irradiation, Sulfhydryl group, Polarographic pattern, Electron spin resonance absorption, Serum albumin, <i>Imai</i>	85
Gas-Chromatographic: — analysis, Fatty acid, Fatty liver, <i>Takahashi and Tanaka</i>	713
Gingival: — Pigmentation, <i>Kuroda, Karazumi, Fujino, Tani, Nagai and Matsuzawa</i>	693
Globulin: bovine —, Chromatographic resolution, Valylleucyl, Methionyl chain, <i>Sasakawa</i> ...	200
γ -Globulin: Biosynthesis, Antibody, Microsome, Immunized Spleen Cell, <i>Ogata, Omori and Hirokawa</i>	660
Gluconolactonase: Lactonase, soluble —, — I, Aldonolactonase, <i>Suzuki, Kawada and Shimazono</i>	448
Glucose: C ¹⁴ — incorporation, <i>Charonia lampas, Iida</i>	173
—: Glutamic acid, Bacteria, Oxidative Metabolism, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio, Ôtsuka and Takahashi</i>	397
—: Estradiol, Nucleotide(s), Bicarbonate, Metabolism, Malonate, <i>Hosoya and Kawada</i>	728
Glutamate: — forming bacterium, <i>Brevibacterium flavum</i> No. 2247, Glyoxylate by-pass enzyme, <i>Shiio, Ôtsuka and Takahashi</i>	262
Glutamic acid: — — γ -hydrazide, Trypsin, <i>Ebata</i>	110
—: Bacterial formation, C ¹⁴ -labelled Acetic acid, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio and Tsunoda</i>	141
—: Bacterial formation, Acetate, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio and Tsunoda</i>	148
—: — formation, glucose, Bacteria, Oxidative Metabolism, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio, Ôtsuka and Takahashi</i>	397
Glutaric acid: <i>Pseudomonas bacteria</i> , <i>Ichihara and Miyake</i>	76
Glutaric semialdehyde: — — dehydrogenase, L-lysine, Bacterial enzyme, <i>Ichihara and Ichihara</i>	154
Glycerate 2,3-diphosphatase: Muscle —, Activation, Mercuric ion, <i>Hashimoto, Nakao and Yoshikawa</i>	595

G (Cont'd)

- Glycine: Formation from Serine, *Muramatsu, Nagayama and Shimura*..... 55
- : — $-C^{14}$, incorporation into protein, Fibroin, *Suto and Shimura*..... 69
- Glycogen: — phosphorylase activity, Tumor, Liver, *Inoue and Oikawa*..... 303
- Glycolysis: Aerobic and Anaerobic —, metabolism, Rat-Ascites-tumor, Nitrogen mustard, Strain, *Miura, Okamoto and Katayama*..... 502
- Glycoprotein: urinary —, N-formylhexosamine, *Hakomori and Ishimoda*..... 264
- γ -Glycoprotein: Abnormal, Cancer, *Hakomori, Ishimoda, Kawauti and Eidoh*..... 307
- Glyoxylate: — by-pass enzyme, Glutamate forming bacterium, *Brevibacterium flavum* No. 2247, *Shio, Otsuka and Takahashi*..... 262
- Guanidino: — compound, γ -Hydroxyarginine, Sea-cucumber, Arginase, Arginine Decarboxylase, *Fujita*..... 468
- Guanidino compound: Sea-anemone, *Anthopleura japonica* Verrill, *Makisumi*..... 284

H

- Haem: Mitochondria, *in vitro*, Protoporphyrin, *Rimington and Tooth*..... 456
- Hemeprotein: Histidine residue, Photooxidation, Catalase, *Nakatani*..... 98
- Hemoglobin: dissociation of —, Urea, *Take...* 206
- : intravenous administration of —, Hemoglobin, Urine, *Kikuchi and Rokugo*..... 446
- : X-ray irradiation, *Kajita, Hashimoto, Okazaki and Kaziro*..... 538
- Hemophilus pertussis*: Cellular components, Isolation, Toxic Lipopolysaccharide, *Kitagawa, Hazama, Motomura, Yamamura, Sawada and Nagai*..... 477
- Hepatoma: metabolism, Rat-Ascites-tumor, Enzyme, Cell, Liver, *Miura, Furiya, Aso, Imadome, Nishimura and Urushibara*..... 519
- : Rat Ascites —, Immunological, Proteins, *Hirai, Sekine, Iijima and Warabioka*..... 682
- Hexanol-celite: — column, assay of inorganic phosphate, *Ohnishi and Ohnishi*..... 83
- L-Hexonate: TPN — Dehydrogenase, Enzymic, Liver, *Mano, Suzuki, Yamada and Shimazono*... 618
- Hexosamine: N-formyl—, *Hakomori and Ishimoda*..... 264
- High-voltage paper Electrophoresis: Iodine, Thyroid gland, *Miyamoto and Kobayashi*..... 548
- Histidine: — residue, Hemeprotein, Photooxidation, Catalase, *Nakatani*..... 98
- Hydroxyarginine: Decarboxylation, Canavanine, *Escherichia coli*, *Makisumi*..... 292

- γ —: Guanidino, Sea-cucumber, Arginase, Arginine Decarboxylase, *Fujita*..... 468

I

- Immunized: — Spleen Cell, Biosynthesis, Antibody, γ -Globulin, microsome, *Ogata, Omori and Hirokawa*..... 660
- Immunochemical: — Studies, Catalase, Erythrocyte(s), Liver, *Higashi, Yagi and Hirai*..... 707
- Immunological: — analyses, Protein, Rat Ascites Hepatoma, *Hirai, Sekine, Iijima and Warabioka*. 682
- Incorporation: Amino acid, cytochrome c, Bacterial, cell-free system, *Mitsui*..... 411
- : Metabolism, Rat-Ascites-tumor, Nitrogen mustard, Strain, Amino acid, Protein, *In vitro*, *Miura, Momose and Moriyama*..... 514
- Indoleacetic acid: *Tsubokura, Sakamoto and Ichihara*..... 38
- Inhibition: ATP-induced Contraction, Mitochondria, Polyhydroxylic compounds, *Lehninger*. 553
- Inhibitor: Oligomycin, Oxidative Phosphorylation, *Huijing and Slater*..... 493
- (s): Trypsin —, Sera, *Nakamura and Wakeyama*..... 733
- Insulin: Tyrosine residue, Lysozyme, Catalase, *Inada*..... 217
- Intracellular: — distribution, D-Amino acid Oxidase, Liver, *Yagi and Nagatsu-Ishibashi*..... 441
- Invertebrate: Comparative biochemical, Cytochrome, Electron transfer, Fresh-water mussels, *Kawai*..... 427
- In vitro*: — formation, Mitochondria, Protoporphyrin, Haem, *Rimington and Tooth*..... 456
- : metabolism, Rat-Ascites-tumor, Nitrogen mustard, Strain, Incorporation, Protein, *Miura, Momose and Moriyama*..... 514
- Iodine: — containing compounds, High-voltage paper Electrophoresis, Thyroid gland, *Miyamoto and Kobayashi*..... 548
- Irradiation: X-ray —, Hemoglobin, *Kajita, Hashimoto, Okazaki and Kaziro*..... 538
- Isokinetin: 2-N-furfurylaminopurine, *Okumura, Kusaka and Takamatsu*..... 133
- Isolation: — of Toxic Lipopolysaccharide, Cellular components, *Hemophilus pertussis*, *Kitagawa, Hazama, Motomura, Yamamura, Sawada and Nagai*..... 477
- : — of a Prothrombin derivative, Conversion, Prothrombin, Thrombin, DEAE-cellulose Chromatography, *Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa*..... 721
- Isohydrixal: Enzymatic, Reduction, Pyridoxine Metabolism, *Fujioka, Morino and Sakamoto*..... 333
- Isovalthine: Biosynthesis, *Fukutome*..... 444

L

Lactic acid: — bacteria, Alanine, Vitamin B ₆ , <i>Chibata, Yamada, Ito, Furutsu and Utsumi</i>	373
Lactonase: Soluble —, — I, Aldonolactonase, Gluconolactonase, <i>Suzuki, Kawada and Shimazono</i>	448
Leber: — wachsender Ratten, Ubichinon, Diätformen, <i>Wiss und Brubacher</i>	609
Light-Anaerobic conditions: Carboxylic acids Metabolism, Porphyrin Biosynthesis, <i>Rhodo- pseudomonas spheroides, Kikuchi, Abe and Muto</i>	570
Lipogenesis: pathological, Unsaturated Fatty acid, Diabetic, <i>Imai</i>	642
Lipopolysaccharide: Toxic —, Cellular com- ponents, <i>Hemophilus pertussis</i> , Isolation, <i>Kitagawa, Hazama, Motomura, Yamamura, Sawada and Nagai</i>	477
Liver: regenerating —, suckling rat —, Tumor, Glycogen phosphorylase activity, <i>Inoue and Oikawa</i>	303
—: rat —, Intracellular, D-Amino acid Oxidase, <i>Yagi and Nagatsu-Ishibashi</i>	441
—: normal —, regenerating —, metabolism, Rat-Ascites-tumor, Enzyme, Hepatoma, <i>Miura, Furiya, Aso, Imadome, Nishimura and Urushibara</i> . .	519
—: rat —, Enzymic, TPN L-Hexonate Dehy- drogenase, <i>Mano, Suzuki, Yamada and Shimazono</i> . .	618
—: Immunochemical, Catalase, Erythrocytes(s), <i>Higashi, Yagi and Hirai</i>	707
Lysine: — Biosynthesis, DL- α -Aminoadipic Acid- 6-C ¹⁴ , <i>T. utilis, Sagisaka and Shimura</i>	392
L—: Bacterial enzyme, Glutaric semialdehyde dehydrogenase, <i>Ichihara and Ichihara</i>	154
Lysophosphatidylethanol-amine: Enzymatic for- mation, Phospholipid, <i>Matsumoto</i>	32
Lysozyme: Tyrosine residue, Insulin, Catalase, <i>Inada</i>	217

M

Malonate: Estradiol, Nucleotide(s), Bicarbonate, Metabolism, Glucose, <i>Hosoya and Kawada</i>	728
Maltoside: Phenyl- α —, Enzymatic hydrolysis, <i>Taka-amylase A, Matsubara</i>	232
Mannitol: — formation, Enzymic, <i>Piricularia oryzae, Yamada, Okamoto, Kodama, Noguchi and Tanaka</i>	404
Mechanism: Anaerobic, Succinate, <i>Proteus vulgaris, Sasaki, Kasamaki and Usami</i>	421
—: — of Transpyrophosphorylation, Biosyn- thesis, Thiamine diphosphate, <i>Kaziro, Tanaka, Mano and Shimazono</i>	472
Melanization: Tyrosinase Activity, melano- some(s) (Melanine Granules), <i>Seiji and</i>	

<i>Fitzpatrick</i>	700
Melanosome(s) (Melanin Granules), Melanization, Tyrosinase Activity, <i>Seiji and Fitzpatrick</i>	700
Mercuric ion: Muscle Glycerate 2, 3-diphos- phatase, Activation, <i>Hashimoto, Nakao and Yoshikawa</i>	595
Metabolic: — Fate, Lysine Biosynthesis, DL- α - Amino adipic acid-6-C ¹⁴ , <i>T. utilis, Sagisaka and Shimura</i>	392
Metabolism: Oxidative —, Glutamic acid, Glucose, Bacteria, <i>Brevibacterium flavum</i> No. 2247, <i>Shiio, Otsuka and Takahashi</i>	397
—: Rat-Ascites-tumor, Nitrogen mustard, Strain, Aerobic, Anaerobic Glycolysis, <i>Miura, Okamoto and Katayama</i>	502
—: Rat-Ascites-tumor, Nitrogen mustard, Strain, <i>Miura, Okamoto and Goto</i>	508
—: Rat-Ascites-tumor, Nitrogen mustard, Strain, Incorporation, Amino acid, Protein, <i>In vitro</i> , <i>Miura, Momose and Moriyama</i>	514
—: Rat-Ascites-tumor, Enzyme, Cell, Liver, Hepatoma, <i>Miura, Furiya, Aso, Imadome, Nishimura and Urushibara</i>	519
—: Carboxylic acids —, Porphyrin Biosynthesis, <i>Rhodopseudomonas spheroides</i> , Light-Anaerobic, <i>Kikuchi, Abe and Muto</i>	570
—: Estradiol, Nucleotide(s), Bicarbonate, Glu- cose, malonate, <i>Hosoya and Kawada</i>	728
Methionine: Enzymatic Synthesis, Methyl group, Spectral, Electrophoretic, Prosthetic group, B ₁₂ Enzyme, <i>Takeyama and Buchanan</i>	578
Methionyl chain: Chromatographic resolution, Globulin, Valylleucyl chain, <i>Sasakawa</i>	200
Methyl group: Enzymatic Synthesis, Methionine, Spectral, Electrophoretic Prosthetic group, B ₁₂ Enzyme, <i>Takeyama and Buchanan</i>	578
Microsome: Biosynthesis, Antibody, γ -Globulin, Immunized Spleen Cell, <i>Ogata, Omori and Hirokawa</i>	660
Mitochondria: brain —al, Noradrenaline, <i>Imamoto and Nukada</i>	177
—: <i>In vitro</i> , Protoporphyrin, Haem, <i>Rimington and Tooth</i>	456
—: Inhibition, ATP-induced Contraction, Poly- hydroxylic compounds, <i>Lehninger</i>	553
—: — structure, <i>Green and Oda</i>	742
Muscle: — Glycerate 2, 3-Diphosphatase, Acti- vation, Mercuric ion, <i>Hashimoto, Nakao and Yoshikawa</i>	595
<i>Mycobacterium</i> : Protein fraction, Phosphorus, <i>Tsukamura</i>	158

N

N-Formylhexosamine: Formyl-residue, <i>Hakomori</i>	
-----------------------------------------------------	--

N (Cont'd)

- and Ishimoda..... 264
- 2-N-furfurylamino-purine: Synthesis, Isokinetin, Okumura, Kusaka and Takamatsu..... 133
- Nereis diversicolor* O. F. Müller: Amino-acide, Euryhalinité, *Perinereis cultrifera* Gr. *Jeuniaux*, Duchâteau-Bosson et Florkin..... 527
- Nitrogen: — -containing phosphate ester, Chromatographic isolation and identification, Horie..... 116
- mustard: metabolism, Rat-Ascites-tumor, Strain, Aerobic, Anaerobic Glycolysis, Miura, Okamoto and Katayama..... 502
- : metabolism, Rat-Ascites-tumor, Strain, Miura, Okamoto and Goto..... 508
- : Metabolism, Rat-Ascites-tumor, Strain, Incorporation, Amino acid, Protein, *In vitro*, Miura, Momose and Moriyama..... 514
- Noradrenaline: brain Mitochondrial particles, Imamoto and Nukada..... 177
- Nori: Chromoprotein, *Porphyra tenera*, Sugar component, Phycocerythrin, Fujiwara..... 361
- Nucleotide(s): Estradiol, Bicarbonate, metabolism, Glucose, Malonate, Hosoya and Kawada. 728

O

- Oligomycin: Inhibitor, Oxidative Phosphorylation, Huijing and Slater..... 493
- Oxidase: Anthranilate —, Cofactor requirement, Hosokawa, Nakagawa and Takeda..... 355
- : Cytochrome —, Activation, *Pseudomonas*, Catalase, Yamanaka, Ota and Okunuki..... 414
- : D-Amino acid —, Intracellular, Liver, Yagi and Nagatsu-Ishibashi..... 441
- Oxidation: — of reduced diphosphopyridine nucleotide, Matsunaka..... 317
- Oxidative: — Metabolism, glutamic acid, glucose, *Brevibacterium flavum* No. 2247, Shio, Otsuka and Takahashi..... 397
- : — Phosphorylation, Oligomycin, Inhibitor, Huijing and Slater..... 493
- Oxygen: —uptake, *Rhodopseudomonas palustris*, Katoh..... 126

P

- Pancreas: —tic lipase, Nishida..... 169
- Papain: Para-thiocresol treatment, Soejima and Shimura..... 260
- Para-thiocresol: — treatment, papain, Soejima and Shimura..... 260
- Pathological: — conditions, Lipogenesis Unsaturated Fatty acid, Diabetic, Imai..... 642
- Pepsin: Tokuyasu and Funatsu..... 297
- Perinereis cultrifera* Gr.: Amino-acide, Euryhalinité *Nereis diversicolor* O. F. Müller, *Jeuniaux*, Duchâteau-Bosson et Florkin..... 527
- Phenyl- α -malto-side: Enzymatic hydrolysis, Taka-amylase A, Matsubara..... 232
- Phosphagen: Dephosphorylative decomposition, Zirconyl ion, Sakurada..... 91
- Phosphate: Photochemical transfer, Mitsui, Ohta and Takashima..... 59
- : Photochemical transfer, green leaves, Mitsui and Ohta..... 65
- : assay of inorganic —, Hexanol-celite column, Ohnishi and Ohnishi..... 83
- : — turnover, nitrogen containing — ester, Chromatographic isolation and identification, Horie..... 116
- Phosphocreatine: Dephosphorylative decomposition, Zirconyl ion, Sakurada..... 91
- Phospholipase: —activity, *Clostridium perfringens* toxin, Phospholipid, Matsumoto..... 23
- Phospholipid: Matsumoto..... 11
- : Phospholipase activity, *Clostridium perfringens* toxin, Matsumoto..... 23
- : Enzymatic formation of Lysophosphatidylethanol-amine, Matsumoto..... 32
- Phosphomevalonsäure-Kinase: Pyrophosphomevalonsäure-Decarboxylase, Biosynthese, Terpene XIV, Tada und Lynen. 758
- Phosphorus: acid soluble — compound Chromatographic isolation and identification, Horie. 116
- : labile — compound, *Mycobacterium*, Tsukamura..... 138
- Phosphorylase: Glycogen — activity, Tumor, Inoue and Oikawa..... 303
- Phosphorylation: Oxidative —, Oligomycin, Inhibitor, Huijing and Slater..... 493
- Photooxidation: — of Catalase, Histidine residue, Heme-protein, Nakatani..... 98
- Phycocerythrin: Chromoprotein, Nori, *Porphyra tenera*, Sugar component, Fujiwara..... 361
- Pigmentation: Gingival —, Kuroda, Karazumi, Fujino, Tani, Nagai and Matsuzawa..... 693
- Piricularia oryzae*: Enzymic, Mannitol, Yamada, Okamoto, Kodama, Noguchi and Tanaka..... 404
- Polarography: —ic pattern, Gamma ray irradiation on protein, Sulfhydryl group, Electron spin resonance absorption, Serum albumin, Imai. 85
- Polyhydroxylic: — compounds, Inhibition, ATP-induced Contraction, mitochondria, Lehninger..... 553
- Porphyra tenera*: Chromoprotein, Nori, sugar component, phycocerythrin, Fujiwara..... 361
- Porphyrin: — Biosynthesis, Carboxylic acids Metabolism, *Rhodopseudomonas spheroides*, Light-Anaerobic, Kikuchi, Abe and Muto..... 570

P (Cont'd)

- Prosthetic group: Enzymatic synthesis, Methyl group, Methionine, Spectral, Electrophoretic, B₁₂ Enzyme, *Takeyama and Buchanan*..... 578
- Protamine: Fractionation of Clupeine, *Ando and Sawada*..... 252
- Protein: Gamma ray irradiation, Sulfhydryl group, Polarographic pattern, Electron, spin resonance absorption, Serum albumin, *Imai*.... 85
- : — fraction of *Mycobacterium*, *Tsukamura*.... 138
- : Acidic and Basic —, *Nagumo*..... 379
- : Serum —, Ultraviolet Densitometry, Agar Electrophoretic, *Shimao*..... 451
- : metabolism, Rat-Ascites-tumor, Nitrogen mustard, Strain, Incorporation, Amino acid, *In vitro*, *Miura, Momose and Moriyama*..... 514
- : Immunological, Rat-Ascites Hepatoma, *Hirai, Sekine, Iijima and Warabioka*..... 682
- Proteus vulgaris*: mechanism, Anaerobic, Succinate, *Sasaki, Kasamaki and Usami*..... 421
- Prothrombin: Conversion of —, DEAE-cellulose Chromatography of —, Isolation of — derivative, Thrombin, *Asada, Masaki, Kitahara, Nagayama, Hatashita and Yanagisawa*..... 721
- Protoporphyrin: C¹⁴-labeled —, Erythrocytes, *Kawai, Sugita and Yoneyama*..... 80
- : Mitochondria, *In vitro*, Haem, *Rimington and Tooth*..... 456
- Pseudomonas*: activation of Glutaric acid, *Ichi-hara and Miyake*..... 76
- : — Cytochrome Oxidase, Activation, Catalase, *Yamanaka, Ota and Okunuki*..... 414
- Pyridoxine: enzymatic reduction of Isopyridoxal, *Fujioka, Morino and Sakamoto*..... 333
- : *Nakahara, Morino, Morisue and Sakamoto*..... 339
- : Fat and Fatty acid administration, *Nakahara, Watanabe, Morino and Sakamoto*..... 343
- Pyrophosphomevalonsäure-Decarboxylase: Phosphomevalonsäure-kinase, Biosynthesis, Terpene XIV, *Tada und Lynen*..... 758
- : Metabolism, Nitrogen mustard, Strain, Incorporation, Amino acid, Protein, *In vitro*, *Miura, Momose and Moriyama*..... 514
- : Metabolism, Enzyme, Cell, Liver, Hepatoma, *Miura, Furiya, Aso, Imadome, Nishimura and Urushibara*..... 519
- Reductase: Sulfite —, Sulfate-reducing bacteria, *Ishimoto and Yagi*..... 103
- : Alcohol-cytochrome-553 —, Alcohol dehydrogenase, Acetic acid bacteria, *Nakayama*.... 240
- Reduction: Sulfate —, Sulfur metabolism in higher plants, *Kawashima and Asahi*..... 52
- : Enzymatic —, Isopyridoxal, Pyridoxine metabolism, *Fujioka, Morino and Sakamoto*..... 333
- Rhodopseudomonas palustris*: Oxygen-uptake by Cell-free extract, *Katoh*..... 126
- : Carboxylic acids Metabolism, Porphyrin Biosynthesis, Light-Anaerobic, *Kikuchi, Abe and Muto*..... 570
- Ribonuclease: — T₁, Chromatographic purification, properties, *Takahashi*..... 1
- : — T₁, *Ui and Tarutani*..... 9
- RNA: Amino acid, Yeast, Alkaline treatment, *Akashi and Ishihara*..... 481

S

- S³⁵: —sulfate, xanthurenic acid, *Sato, Fukuyama, Suzuki, Yoshikawa and Gorrod*..... 164
- Sea-anemone: *Anthopleura japonica* Verrill, Guanidino compound, *Makisumi*..... 284
- Sea-cucumber: γ -Hydroxyarginine, Guanidino, Arginase, Arginine Decarboxylase, *Fujita*..... 468
- Sedative: α -Bromoisovalerylurea (Bromural), *Haruna*..... 388
- Sera: Trypsin Inhibitor(s), *Nakamura and Wakeyama*..... 733
- Serin: formation of Glycine, *Muramatsu, Nagayama and Shimura*..... 55
- Serotonin O-sulfate: Synthesis, *Kishimoto, Takahashi and Egami*..... 436
- Serum: bovine — albumin, Gamma ray irradiation on protein, Sulfhydryl group, Polarographic pattern, Electron spin resonance absorption, *Imai*..... 85
- : — Proteins, Ultraviolet Densitometry, Agar Electrophoretic, *Shimao*..... 451
- : — Caeruloplasmin, Determination, *Shimizu, Maruyama, Kukita, Yanagisawa, Sato and Osaki*. 673
- Soluble: — Lactonase, Lactonase I, Aldonolactonase, Gluconolactonase, *Suzuki, Kawada and Shimazono*..... 448
- Spectral: Enzymatic Synthesis, Methyl group, Methionine, Electrophoretic, Prosthetic group, B₁₂ Enzyme, *Takeyama and Buchanan*..... 578

R

- Radioactivation: — Analysis, Strontium, Bone ash, *Matsumura and Fujino*..... 561
- Radioactive: — Tin, *Morimoto and Yoneyama*.... 601
- Rana catesbiana*: Synthesis of stero-bile acid, Bile acid, Cholesterol, *Masui*..... 211
- Rat Ascites Hepatoma: Immunological, Proteins, *Hirai, Sekine, Iijima and Warabioka*..... 682
- Rat-Ascites-tumor: Metabolism, Nitrogen mustard, Strain, Aerobic, Anaerobic Glycolysis, *Miura, Okamoto and Katayama*..... 502
- : Metabolism, Nitrogen mustard, Strain, *Miura, Okamoto and Goto*..... 508

S (Cont'd)

Spleen: Immunized — Cell, Biosynthesis, Antibody, γ -Globulin, Microsome, <i>Ogata</i> , <i>Omori</i> and <i>Hirokawa</i>	660
Stero-bile acid: formation of Bile acid, Cholesterol, <i>Rana catesbiana</i> , <i>Masui</i>	211
Steroidsulfatase: <i>Charonia lampas</i> , liver, <i>Takahashi</i> and <i>Egami</i>	583
Strain: sensitive and resistant —, Metabolism, Rat-Ascites-tumor, Nitrogen mustard, Aerobic, Anaerobic Glycolysis, <i>Miura</i> , <i>Okamoto</i> and <i>Katayama</i>	502
—: Nitrogen mustard sensitive and resistant —s, Metabolism, <i>Miura</i> , <i>Okamoto</i> and <i>Goto</i>	508
—: Nitrogen mustard sensitive and resistant —s, Metabolism, Rat-Ascites-tumor, Incorporation, Amino acid, Protein, <i>In vitro</i> , <i>Miura</i> , <i>Momose</i> and <i>Moriyama</i>	514
<i>Streptococcus</i> : —al ghost, Streptolysin S' formation, <i>Sugai</i>	348
Streptolysin: —S', Streptococcal ghost, <i>Sugai</i> ...	348
Strontium: Radioactivation Analysis, Bone ash, <i>Matsumura</i> and <i>Fujino</i>	561
Succinate: mechanism, Anaerobic, <i>Proteus vulgaris</i> , <i>Sasaki</i> , <i>Kasamaki</i> and <i>Usami</i>	421
Sugar: — component, Chromoprotein, <i>Nori</i> , <i>Porphyra tenera</i> , Phycoerythrin, <i>Fujiwara</i>	361
Sulfate: — reduction, Sulfur metabolism in higher plants, <i>Kawashima</i> and <i>Asahi</i>	52
—: —reducing bacteria, Sulfite reductase, <i>Ishimoto</i> and <i>Yagi</i>	103
—: S ³⁵ —, conjugation with phenolic compound, xanthurenic acid, <i>Sato</i> , <i>Fukuyama</i> , <i>Suzuki</i> , <i>Yoshikawa</i> and <i>Gorrod</i>	164
Sulphydryl group: Gamma ray irradiation on protein, Polarographic pattern, Electron spin resonance absorption, Serum albumin, <i>Imai</i>	85
Sulfite: — reductase, Sulfate-reducing bacteria, <i>Ishimoto</i> and <i>Yagi</i>	103
Sulfur: — metabolism, Sulfate reduction, <i>Kawashima</i> and <i>Asahi</i>	52
Synthesis: Serotonin O-sulfate, <i>Kishimoto</i> , <i>Takahashi</i> and <i>Egami</i>	436
—: Enzymatic —, methyl group, methionine, Spectral, Electrophoretic, Prosthetic group, B ₁₂ Enzyme, <i>Takeyama</i> and <i>Buchanan</i>	578

T

<i>T. utilis</i> : Lysine Biosynthesis, Metabolic, DL- α -Aminoadipic acid-6-C ¹⁴ , <i>T. utilis</i> , <i>Sagisaka</i> and <i>Shimura</i>	392
Taka-amylase: —A, <i>Takagi</i> and <i>Isemura</i>	43
—: —A, Trans-maltosidation, <i>Matsubara</i>	226
—: —A, enzymatic hydrolysis, Phenyl- α -malt-	

oside, <i>Matsubara</i>	232
—: —A, Urea denaturation, kinetic aspect, <i>Fujita-Ikeda</i>	267
—: —A, Urea denaturation, size and shape, <i>Isemura</i> and <i>Fujita-Ikeda</i>	278
Terpene: Phosphomevalonsäure-Kinase, Pyrophosphomevalonsäure-Decarboxylase, Biosynthesis, <i>Tada</i> and <i>Lynen</i>	642
Thiamine: diphosphate: Mechanism, Transpyrophosphorylation, Biosynthesis, <i>Kaziro</i> , <i>Tanaka</i> , <i>Mano</i> and <i>Shimazono</i>	472
—: Treatment Dosis, <i>Shibata</i> , <i>Yamashita</i> and <i>Yasuda</i>	589
Thrombin: Conversion, Prothrombin, DEAE-cellulose Chromatography, Isolation, <i>Asada</i> , <i>Masaki</i> , <i>Kitahara</i> , <i>Nagayama</i> , <i>Hatashita</i> and <i>Yanagisawa</i>	721
Thyroid gland: High-voltage paper Electrophoresis Iodine, <i>Miyamoto</i> and <i>Kobayashi</i>	548
Tin: Radioactive —, <i>Morimoto</i> and <i>Yoneyama</i> ...	601
Toxic: — Lipopolysaccharide, Cellular components, <i>Hemophilus pertussis</i> , Isolation, <i>Kitagawa</i> , <i>Hazama</i> , <i>Motomura</i> , <i>Yamamura</i> , <i>Sawada</i> and <i>Nagai</i>	477
Toxicity: Oil(s), Cyclic, β -Eleostearic acid, <i>Matsuo</i>	635
Toxin: <i>Clostridium perfringens</i> —, Phospholipase activity, Phospholipid, <i>Matsumoto</i>	23
TPN: See Triphosphopyridine nucleotide	
Transmaltosidation: Taka-amylase A, <i>Matsubara</i>	226
Transpyrophosphorylation: Mechanism of —, Biosynthesis, Thiamine diphosphate, <i>Kaziro</i> , <i>Tanaka</i> , <i>Mano</i> and <i>Shimazono</i>	472
Treatment Dosis: Thiamine, <i>Shibata</i> , <i>Yamashita</i> and <i>Yasuda</i>	589
Triphosphopyridine nucleotide: preparation from pig liver, <i>Yamada</i> , <i>Koike</i> , <i>Yamada</i> and <i>Tanaka</i>	353
— —: TPN, L-Hexonate dehydrogenase, Enzymic, Liver, <i>Mano</i> , <i>Suzuki</i> , <i>Yamada</i> and <i>Shimazono</i>	618
Trypsin: action upon Glutamic acid- γ hydrazide derivative, <i>Ebata</i>	110
—: — Inhibitor(s), <i>Sera</i> , <i>Nakamura</i> and <i>Wakeyama</i>	733
Tumor: Glycogen phosphorylase activity, <i>Inoue</i> and <i>Oikawa</i>	303
Tyrosinase: — Activity, Melanization, Melanosome(s) (Melanin Granules), <i>Seiji</i> and <i>Fitzpatrick</i>	700
Tyrosine: — residue, Insulin, lysozyme, Catalase, <i>Inada</i>	217

U

Ubichinon: — gehalt, Leber, Diätformen, <i>Wiss</i>	
-----------------------------------------------------	--

U (cont'd)

<i>und Brubacher</i>	609
Ultraviolet: — densitometry, Serum Proteins, Agar Electrophoretic, <i>Shimao</i>	451
Unsaturated: — Fatty acid, Lipogenesis, patho- logical, Diabetic, <i>Imai</i>	642
Urea: action of —, dissociation of Hemoglobin, <i>Take</i>	206
—: — denaturation, Taka-amylase A, <i>Fujita- Ikeda</i>	267
—: — denaturation, Taka-amylase A, <i>Isemura and Fujita-Ikeda</i>	278
Uridine diphosphoglucuronic acid: quantitative determination, <i>Kawada</i>	78
Urine: Choleglobin, Hemoglobin, <i>Kikuchi and Rokugo</i>	446

V

Valylleucyl chain: Chromatographic resolution, Globulin, Methionyl chain, <i>Sasakawa</i>	200
Vitamin B ₆ : Alanine, Lactic acid bacteria, <i>Chibata, Yamada, Ito, Furutsu and Utsumi</i>	373

X

Xanthurenic acid: conjugation of S ³⁵ -Sulfate with phenolic compound, <i>Sato, Fukuyama, Suzuki, Yoshikawa and Gorrod</i>	164
----------------------------------------------------------------------------------------------------------------------------------------------------	-----

Y

Yeast: Amino acid, RNA, Alkaline treatment, <i>Akashi and Ishihara</i>	481
---------------------------------------------------------------------------------	-----

Z

Zirconium: —yl ion, Phosphagen, Phospho- creatine, <i>Sakurada</i>	91
-----------------------------------------------------------------------------	----

